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CANCER RESEARCH

VOL. 10 AUGUST 1950 No. 8

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THE OFFICIAL ORGAN OF THE

AMERICAN ASSOCIATION FOR CANCER RESEARCH, INC.

Published by THE UNIVERSITY OF CHICAGO PRESS

CANCER RESEARCH

This journal is sponsored by The American Association for Cancer Research, Inc.; The Anna Fuller Fund; Cancer Research Division, Donner Foundation, Inc.; The Jane Coffin Childs Memorial Fund for Medical Research; The Elsa U. Pardee Foundation; and The American Cancer Society.

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For the British Empire, except North America and Australasia:

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Business communications, remittances (in United States currency or its equivalent), and subscriptions should be addressed to THE UNIVERSITY OF CHICAGO PRESS, 5750 Ellis Avenue, Chicago 37, Illinois. All other communications should be addressed to Harold P. Rusch, M.D., McArdle Memorial Laboratory, University of Wisconsin, Madison 6, Wisconsin.

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Entered as second-class matter, February 15, 1949, at the post office at Chicago, Ill., under the Act of March 3, 1879.

Acceptance for mailing at special rate of postage provided for in United States Postal Act of October 3, 1917, Section 1103, amended February 28, 1925, authorized June 1, 1950.

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CANCER RESEARCH

VOLUME 10 AUGUST 1950 NUMBER 8

Recent Studies on Corneal Metabolism and Growth: *A Review*

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There is hardly a treatise on oncology that does not begin with the definition of cancer as a tissue or group of cells that has in some degree escaped from the "normal" restraints of tissue growth and is growing "autonomously." One searches in vain, however, for any clear insight into the nature of these normal restraints and their mode of operation. The problem is touched upon tangentially in the field of hormone research and is central to developmental embryology where, unfortunately, the issue has been beclouded by the somewhat mythical concept of organizers. No disparagement is meant here of the truly monumental work of the past generation in the field of experimental embryology, for in the embryo everything is growing and differentiating and changing simultaneously, so that there is no fixed point of reference from which the factors governing the changes in a particular group of cells can be evaluated. It was most natural to assume that these multitudinous effects might be mediated by some specific organizers, and this possibility has not been definitely excluded even by a generation of unsuccessful attempts to isolate and characterize such organizers.

More recent trends of thought indicate a growing awareness of the multiplicity of possible metabolic interactions of adjacent cells and tissues, and a theoretic basis for differentiation resulting from such interactions has been provided by Spiegelman (42). Drawing implications from his work on yeast, Spiegelman has suggested that cells acquire their characteristic pattern of enzymes by virtue not only of their genetic potentialities but also, specifically, under the influence of available substrates or metabolites; that due to substrate gradients in the embryo, cells in different locations acquire different enzyme content; and that in this differentiation they further alter each other's environment so as to lead to still further differentiation. It would seem that the test of Spiegelman's hypothesis would be most difficult in the field of embryonic development, where everything is changing at once, and might be pursued more fruitfully in relation to adult tissues, where everything except those factors under specific observation can be kept in relatively stable homeostatic balance.

It is in the perspective of these broad questions that some of the recent work on the cornea is to be reviewed. This tissue is perhaps one of the simplest organized structures in the mammalian body, but even the preliminary and halting steps that have so far been taken in its study reveal a high degree of interaction of its neighboring components. At this early stage no very general and well defined patterns have as yet become visible, but even these small beginnings may serve to stimulate similar studies on other tissues and to make clear how long is the road ahead toward an understanding of the normal restraints of tissue growth which lead to orderly and organized structure, and from which cancer cells somehow escape into "autonomous" growth.

Metabolic organization.—The main bulk of the cornea is the collagenous stroma sparsely supplied with cells. This is covered on its anterior surface by a layer of epithelium four to six cells deep, and on its posterior surface by a single layer of mesothelium. Since the epithelium or mesothelium can be scraped off with little damage to the stroma, it is possible to compare the metabolism of the separated layers with that of the intact tissue.

Received for publication, April 29, 1950.

Herrmann and his co-workers (27–30) found that the epithelium contains glycogen, has a cyanide-sensitive respiration, and a conventional glycolytic and glucolytic mechanism. The corneal stroma, on the other hand, has no oxygen uptake but consumes as much glucose per cell as does the epithelium. The product of this glucose consumption is lactic acid. In the intact cornea the lactic acid produced by the stroma is consumed by the epithelium and constitutes one-quarter or more of the total carbohydrate utilized by these cells.

The denuded stroma, on incubation, rapidly loses its capacity to glucolyze, the process coming to an end when all the available inorganic phosphate has been incorporated into triose phosphate. It is evident that this tissue lacks a phosphate acceptor, presumably adenosine diphosphate, needed for the conversion of triose phosphate to lactate, and for the continued operation of the glucolytic cycle. Thus, the metabolic relation between the epithelium and stroma is a reciprocal one, in which the epithelium receives lactate and simultaneously delivers to the stroma a necessary co-factor for glucolysis.

There is some evidence suggesting that the transfer of lactate from stroma to epithelium is an active one, not simply passive diffusion. Butyrate and serine injected into the stroma are not utilized by that denuded tissue but in the intact structure are consumed at approximately the same rate as lactate. The utilization of stroma lactate, butyrate, and serine by the epithelium are all inhibited by the same concentration of mustard gas. It would appear that the stroma-epithelium boundary is the locus of highly complex metabolic activity.

Cogan and Kinsey (8, 9) have shown that this boundary has highly specialized permeability characteristics, being permeable to water and to lipoid-soluble substances but relatively impermeable to crystalloids. There is no necessary contradiction between the active transport of certain substances across a boundary and the impermeability of that boundary to diffusion of these same or similar substances. In fact, if active transport is to be effective, it must be associated with an impermeability to diffusion of the transported substance, since otherwise back diffusion would very greatly diminish the net amount of substance transported. The mechanism of active transport in this case has not been investigated.

Tissue cohesion.—An important aspect of the interrelation of adjacent tissues is that of cohesion. It would appear that this attribute is not without significance for some aspects of invasiveness of tumors and for the relation of some tumors to their own stroma. This subject has in the past received only scattered attention. The general literature in this field has been summarized by Buschke (4).

The adhesion of the corneal epithelium to the underlying stroma was studied by Herrmann *et al.* (24–26). Among the substances which cause a loosening of the corneal epithelium are those of detergent type, including compounds of such low molecular weight as butyl alcohol. The authors were led by this finding to the conclusion that the cohesive boundary includes a lipid component. On the other hand, proteolytic enzymes, e.g., trypsin and chymotrypsin, also resulted in loosening of the epithelium, indicating a protein component of the cohesive boundary. Inorganic salts and wide variation in pH had little effect, suggesting that acid-base bonds played no significant role in the cohesive mechanism. Exposure to certain metabolic poisons and subsequent incubation resulted in loosening of the epithelium. Similar results were obtained following exposure to such vesicating agents as freezing and mustard gas. The authors concluded that the maintenance of coherent organization may require metabolic energy, but the possibility that proteolytic enzymes might be activated under these circumstances could not be excluded. Local anesthetics are particularly effective in causing epithelial loosening. Herrmann *et al.* (31) found that local anesthetics were powerful inhibitors of respiration in the corneal epithelium, but this did not explain their influence on tissue cohesion, since cyanide also suppressed respiration but did not result in loosening. Exposure to very low concentrations of histamine resulted in a loosening of the superficial layers of the corneal epithelium, leaving the basal layer still firmly adherent to the stroma.

The cohesion of the corneal epithelial cells to one another has been studied in some detail by Buschke (4, 5). Trypsin, chymotrypsin, and papain greatly decreased the intercellular cohesion, while lysozyme, hyaluronidase, ribonuclease, and crotoxinlipase were without effect. The effect of the proteolytic enzymes was antagonized by calcium and, to a lesser degree, by other alkaline earth elements and by lithium. Iodide and thiocyanate tend to diminish this calcium effect. Anionic detergents were found to abolish intercellular cohesion, but cationic and non-ionic detergents were without significant effect. Buschke has studied the influence of various metabolic poisons on the intercellular cohesion. Exposure to fluoride, iodoacetate, iodoacetamide, 2,4-dinitrophenol and 4,6-dinitrocresol, and some local anesthetics, followed by subsequent incubation, led to a loss of cohesion. Cyanide, malonate, and azide were in-

effective. Quinone and trinitrophenol were very effective, and exposure to these agents led to dissociation of the corneal epithelial sheet into a mass of isolated cells, after only brief incubation. It is evident that there are some similarities but also some major differences between the forces that bind the epithelial cells to one another and the forces that bind the epithelial sheet to the underlying stroma

Mitotic activity.—Studies on the mitotic activity of the corneal epithelium have been undertaken by a considerable number of investigators. The literature in this field has been recently reviewed by Friedenwald (10) and Buschke (3). Many of these studies were undertaken for the immediate, practical purpose of evaluating local toxic effects of the commonly used medications, antibiotics, etc., and have little direct relevance to the cancer problem. A useful and simple method of mitotic assay in this tissue has been worked out by Buschke, Friedenwald, and Fleischmann (6), and has been widely used by other investigators with generally concurrent results.

Mitotic activity in the corneal epithelium is limited to the two most basally located cell layers. In the rat, the animal of choice for most of this work, the process of mitosis in these cells requires approximately 70 minutes. The intermitotic period is approximately 200 hours. This long interphase renders this tissue of some special value for comparison with those others, mostly characterized by quite short interphases, that have been used in the past for quantitative mitotic studies. There are many inhibitors of mitosis whose effect is predominantly or exclusively that of prolonging interphase. In relation to some of these, the normally long interphase of the corneal epithelium renders this tissue especially useful for the investigation of threshold effects. Agents in respect to which the threshold of mitotic inhibition have been fruitfully investigated are mustard gas and related compounds (15, 20, 21), ultraviolet (7, 17) and x-radiation (19, 41), and adrenalin (13).

Pin-prick wounds of the corneal epithelium cause an inhibition of mitosis in the adjacent uninjured cells (12). Cervical sympathectomy diminishes the mitotic rate and also slows down the mitotic cycle (13). Similar effects have been found in vitamin A deficiency (18) and in barbiturate anesthesia (14). Local anesthetics and sympathicomimetic drugs are powerful inhibitors of mitosis (14). Several vitamin deficiencies (ascorbic acid, riboflavin) have been found to be without effect on corneal mitotic activity even when the growth of the animal as a whole has been severely stunted (14). The effect of age has been studied by Smelser (37), but, outside of this, the large and interesting field of endocrine effects has not been explored.

Buschke, Friedenwald, and Moses (7) found that threshold exposures to a low-pressure, quartz mercury vapor arc caused an increase in mitotic activity in the rat's corneal epithelium. Further analysis of this phenomenon (17) revealed that the effective stimulating agent was not the radiation emitted by the arc but some gaseous component, possibly ozone, in the atmosphere surrounding the arc. No stimulating effect was found in an extended investigation of the whole accessible ultraviolet spectrum when the possibility of ozone contamination was excluded. The possible bearing of these findings on the much disputed "mitogenetic" rays of Gurwich deserves consideration.

Agents which stimulate or retard mitotic activity are of interest in that they may furnish clues to the normal restraints on growth by which the orderly relation of the tissues is maintained. Unfortunately, those agents thus far studied have not been particularly illuminating in this respect.

Of more immediate and practical interest to the oncologist are those mitotic poisons that cause death of rapidly growing tissues. The mode of cellular death produced by these agents—nuclear fragmentation or karyorrhexis—has been studied in many organs and tissues. It is of some interest that three of the most extensively studied agents of this type, ionizing radiation, ultraviolet radiation, and the nitrogen mustards (19, 21), show wide differences in this regard on the rat's corneal epithelium. Ionizing radiation, although capable of producing karyorrhexis in many other tissues, fails to produce this effect in the corneal epithelium even when administered in doses up to 8,000 r. The nitrogen mustards produce karyorrhexis in a small number of cells in the basal epithelium. Friedenwald, Buschke, and Scholz (21) have advanced evidence, admittedly indirect, suggesting that the cells so affected are those that were in premitosis at the time of exposure. The dosage of the toxic agent required to produce these fatal injuries is about 1,000 times that which produces a temporary inhibition of mitosis. Ultraviolet radiation in adequate dosage results in karyorrhexis of very great numbers of cells in all layers of the corneal epithelium. Even the superficial cells which are normally devoid of mitotic activity undergo karyorrhexis after exposure to ultraviolet radiation. The action spectrum for this effect has a broad peak in the region of 280 mμ (17). Karyorrhexis in the corneal epithelium shows many morphologic similarities to normal mitosis, including a marked increase in the amount of Feulgen positive

intranuclear material. If it is to be regarded as a pathologic and fatal attempt at mitosis, then ultraviolet radiation must be capable of stimulating the senescent cells of the superficial layers in this tissue to engage in this youthful and, for them, fatal activity. Nucleic acid phosphatase is not inhibited at dosage levels of nitrogen mustard, ultraviolet, or x-radiation which cause marked inhibition of mitosis with or without karyorrhexis (22).

Wound healing.—Much of the work on the healing of corneal wounds has been directed toward practicalities of ocular therapeutics (3, 10). Local anesthetics have a markedly inhibiting effect (25). Leopold and Steele (32, 33) found that even the blandest ointment bases caused slight delay in the healing of denuded areas in the cornea. Their findings have been confirmed by others (38, 39). Friedenwald and Heerema (23) found that the toxic factor in lanolin, a component of most ophthalmic ointments, could be removed by washing the lanolin in phosphate buffer. The toxic factor has not been isolated but might be worthy of further study, for there are some analogies between the invasiveness of certain tumors and the healing of epithelial wounds.

Since the work of Oppel (35) and Peters (36), it has been clear that epithelial wounds heal primarily by the migration of adjacent cells into the denuded area and that cellular multiplication plays only a later and secondary role. The truth of this general rule has been abundantly demonstrated with respect to the corneal epithelium. Mechanical injury actually causes a temporary diminution of mitotic activity (12). If the wounds are very small (pin pricks) they may heal completely during the period of mitotic inhibition. Even with larger wounds there is, in general, no net excess of mitotic activity during or following the healing process. The deficit in cells appears to be made up by a diminished rate of desquamation (14). Smelser and Ozanics, however, found some net excess of mitotic activity during the healing of small thermal burns of the corneal epithelium (40).

Following pin-prick wounds of the rat's corneal epithelium (12), there is a lag period of somewhat more than an hour before the healing process visibly begins. During the second hour after the injury, the marginal cells begin to extend pseudopods into the denuded region and become elongated radially toward the defect. The morphology of these events was studied by Friedenwald, Buschke, and Crowell (16) and later in greater detail by Buschke (1, 2) who has analyzed the separate roles of individual cell movements and of movements of the epithelial syncytium as a unit. The healing of corneal wounds can be studied *in vitro* as well as *in vivo*. It is inhibited by anoxia or by cyanide poisoning but not by fluoride. The capacity of cells to migrate can persist when many other vital phenomena have disappeared. Buschke (2) finds that rat corneas, stored in the cold until the basophilic staining capacity of their epithelial cell nuclei has been completely lost, still show healing of pin-prick wounds at a normal rate when incubated at 37° C. following injury.

The events which occur during the lag period immediately following the injury are of special interest, for it is during this period that the stimulus to cellular migration becomes manifest. Friedenwald, Buschke, and Crowell (16) found that during the lag period an exudate is formed within the pin-prick wound. The formation of this exudate is inhibited on incubation at low temperature or in the absence of oxygen. It is therefore the product of active metabolism. One component of this exudate is extractable with lipoid solvents and stainable with silver. Further studies by Buschke (1, 2) show that this exudate is derived at least in part from the sequestrum of fatally damaged cells which must be sloughed off before the healing can begin. The development of the exudate, therefore, may be merely a step in the process of sloughing and not an active stimulus to subsequent healing.

Growth pressure and metaplasia.—In the healing of wounds of the corneal epithelium which extend to the corneal margin, conjunctival as well as corneal epithelium may participate in covering the defect. In darkly pigmented animals there is generally a line of pigmented cells at the corneal-conjunctival border, and the participation of the conjunctival epithelium in the healing process becomes grossly visible through the shifting onto the cornea of this demarcation line. This phenomenon was first described by Mann and Pullinger (34) in their study of mustard gas burns of the cornea. When the corneal stroma has also been injured and is healed with the formation of a vascularized scar, epithelium of conjunctival type may persist as a peninsula covering the vascularized area. In the absence of such a vascularized scar, the demarcation line slowly returns to its normal position.

This interesting phenomenon has been recently reinvestigated by Friedenwald (11). If the whole corneal epithelium of a rabbit is removed, healing takes place entirely from cells of conjunctival origin. During the first week of healing these cells form a single thin layer extending onto and finally covering the whole cornea. During this phase these

cells contain neither mucous globules that are characteristic of the conjunctival epithelium nor glycogen that is characteristic of normal corneal epithelium. About 2 weeks after the denudation the cells assume the characteristic appearance of conjunctival epithelium, forming a membrane two cells deep, with many goblet cells containing mucin. In the course of the succeeding weeks the epithelial layer is gradually transformed into corneal type, forming a layer four to six cells deep with glycogen and not mucin in their cytoplasm. During the period of transformation, occasional cells can be found containing both glycogen and mucin. Mitosis counts indicate that the transformation is complete when the conjunctival cells have lived in their new environment for two to four generations. In those instances in which infected ulcerations of the cornea occurred during the period of denudation, epithelium of conjunctival type persists over the area of the vascularized scar.

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Studies in Hodgkin's Syndrome

XI. The Influence of Normal Serum and Hodgkin's Serum on Cellular Growth and Morphology in Tissue Culture*

MARGARET S. REIMAN, M.S., IRENE R. STERN, B.S., MARGARET Z. FORD, B.S., AND HERMAN A. HOSTER, M.D.

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A study of the effect of normal human serum and serum from Hodgkin's patients on normal and diseased human cells in tissue culture has been undertaken. The Hodgkin's serum-induced morphologic changes in normal and diseased cells are compared with the morphologic characteristics of Hodgkin's explant cells in the presence of normal serum.

Lustig and Lyonnet (4) conclude that normal serum exhibits an inhibiting action and cancer serum a stimulating action on the growth of normal fibroblasts and myeloblasts *in vitro*. Norris and Majnarich (6), on the other hand, report that normal blood serum accelerates—and blood serum from cases of pernicious anemia, leukemia, and neoplastic disease inhibits—cell proliferation in normal bone marrow cultures *in vitro*, and they note that the opposite effect is observed in human and animal neoplastic tissues other than bone marrow. The accelerating substance of normal serum and the inhibiting substance of neoplastic disease serum are said to counteract each other in normal bone marrow cultures.

Grand (2) has referred briefly to the effect of autogenous Hodgkin's serum on the growth characteristics of Hodgkin's lymph nodes *in vitro*. Liquefaction, degeneration, giant cell formation, and stimulation of reticulum cells are said to occur under these conditions.

MATERIALS AND METHODS

Human lymph nodes and spleens were obtained under sterile conditions at surgery. Part of each specimen was subjected to paraffin sectioning, staining, and histologic examination. The remainder was cultured within 4 hours by means of the roller-tube tissue culture technic (1). Normal human tissues were obtained under sterile conditions from newborn infants at autopsy. Human bone marrow specimens were obtained by sternal aspiration. A differential cell count was done in each case, and the remainder of the marrow was cultured immediately. Human white blood cell samples were removed from whole blood obtained by venipuncture. The whole blood obtained was centrifuged before coagulation; the fluid plasma was removed with a pipette, leaving the layered buffy coat and red blood cells beneath it. The buffy coat layer was then "coagulated" by the addition of one drop of bovine fibrinogen and thrombin, and the clotted white cells were removed *en masse*. The white blood cells were cultured within $\frac{1}{2}$ hour after separation from the whole blood sample.

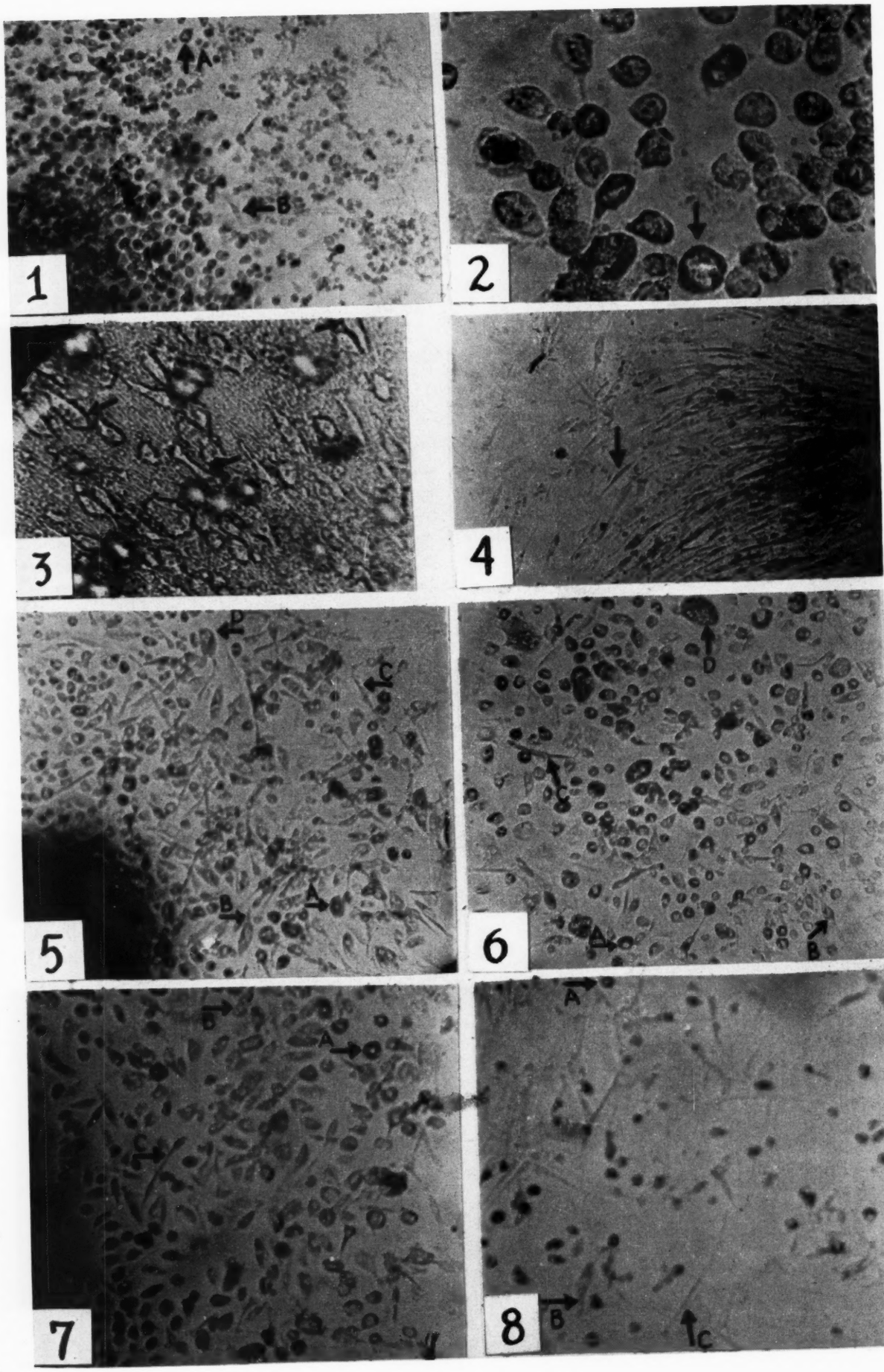
Bovine thrombin and bovine fibrinogen, obtained in powdered form commercially, were used routinely as a clotting medium in all tissue cultures prepared (7). A balanced salt solution (Gey's solution) was sterilized by filtration and added to the nutrient fluid.

Human placental serum (cell-free) was used routinely as a part of the nutrient fluid. The placental blood was obtained from the cord vein following delivery of the placenta. After clotting, the serum was removed in a sterile manner and stored at 4° C. until used. Normal human adult serum (cell-free) was a part of the nutrient fluid in control cultures, and serum obtained from Hodgkin's patients in the intermediate or advanced stage of the disease was a part of the nutrient fluid in experimental cultures. The adult serum was stored at the same temperature as the placental serum.

One hundred and twenty-eight tissues from di-

* This investigation was supported by research grants from the National Cancer Institute, Public Health Service, and the American Cancer Society on recommendation of the Committee on Growth of the National Research Council.

Received for publication January 16, 1950.



FIGS. 1–8*

verse human sources were cultured under the influence of normal serum and of Hodgkin's serum (Table 1).

The roller-tube technic (1) was used routinely in culturing tissues. The tissues were minced with scissors, and six pieces, 0.5–1.0 mm. in size, were imbedded in a fibrinogen and thrombin coagulum at intervals extending from the lower to the upper end of each tube. One cc. of one of the following nutrient fluids was then introduced into each tube: (*a*) Control: 2 parts balanced salt solution, 1 part normal placental serum, and 1 part normal adult serum; (*b*) Experimental: 2 parts balanced salt solution, 1 part normal placental serum, and 1 part Hodgkin's serum.

The tubes, stoppered with rubber "vaccine or serum" bottle tops, were incubated at 37.5° C. in a rotating roller-tube wheel. Each culture was nourished semiweekly with fresh fluid after removal of the used fluid.

Microscopic observations (100 × magnification and 500 × magnification) were made daily on new and rapidly growing cultures and biweekly on old and more slowly growing cultures. Photomicrographs of representative, unstained, living explants within the roller tube were taken weekly, or

TABLE 1

TISSUES STUDIED UNDER THE INFLUENCE OF NORMAL SERUM AND HODGKIN'S SERUM

Diagnosis	Lymph node	Spleen	Thymus	Bone marrow cells	White blood cells	Liver	Lung
Hodgkin's disease	28	3		3	2		
Normal fetal and neonatal (9)	36	9	8			1	1
Nonspecific lymphadenitis	11						
Normal adult (2)					2		
Lymphatic leukemia	2						
Lymphosarcoma	3						
Monoblastic leukemia					1		
Reticulum-cell sarcoma	2						
Metastatic carcinoma	3						
Alveolar carcinoma	1						
Giant follicular lymphoblastoma	3						
Unclassified lymphoma	1						
Carcinoma of the rectum				1			
Plasma-cell myeloma				1			
Sarcoidosis	1	1					
Letterer-Siwe's disease	1						
Neurologic syphilis				1			
Congestive heart failure				1			
Tuberculosis of lymph node	1						
TOTALS:	93	13	8	7	5	1	1

FIG. 1.—Hodgkin's lymph node, type I: granule-containing, free cell, macrophage and reticulum cell type; 13-day-old culture in normal serum. This type is characterized by a free cell form of growth made up predominantly of macrophage-type cells packed with Sudan IV staining bodies. The cell types indicated by arrows are: (*a*) the macrophage-type cell and (*b*) the reticulum cell. Mag. ×300.

FIG. 2.—Hodgkin's lymph node, type I: granule-containing, free cell, macrophage and reticulum cell type; 13-day-old culture in normal serum. The arrow illustrates the macrophage-type cell. Mag. ×1500.

FIG. 3.—Hodgkin's lymph node, type II: granule-free, free cell, "abortive fibroblast" type; 178-day-old culture in normal serum. This type is composed principally of "abortive fibroblast" forms and, like type I, manifests a free cell form of growth. Unlike type I, however, the cells are not packed with fat bodies. The arrows illustrate "abortive fibroblasts." Mag. ×780.

FIG. 4.—Hodgkin's lymph node, type Ia: network or fixed cell fibroblast type; 18-day-old culture in normal serum. This type, unlike types I and II, is for the most part a fixed cell type of growth and contains "true" fibroblasts. The fibroblasts in type Ia, however, grow in looser sheets and with more irregularity than do normal fibroblasts. The arrow illustrates a fibroblast. Mag. ×240.

FIG. 5.—Hodgkin's lymph node, type I: 7-day-old culture in normal serum. The cell types indicated by arrows are: (*a*) the macrophage-type cell, (*b*) the reticulum cell, (*c*) the fibroblast, and (*d*) the giant cell. Mag. ×240.

FIG. 6.—Hodgkin's lymph node, type I: similar in every respect to Fig. 5, except that it has been nourished with Hodgkin's serum rather than normal serum. The most obvious serum-induced effect is an increase in the number of nuclei in the giant cells. The cell types indicated by arrows are: (*a*) the macrophage-type cell, (*b*) the reticulum cell, (*c*) the fibroblast, and (*d*) the giant cell. Mag. ×240.

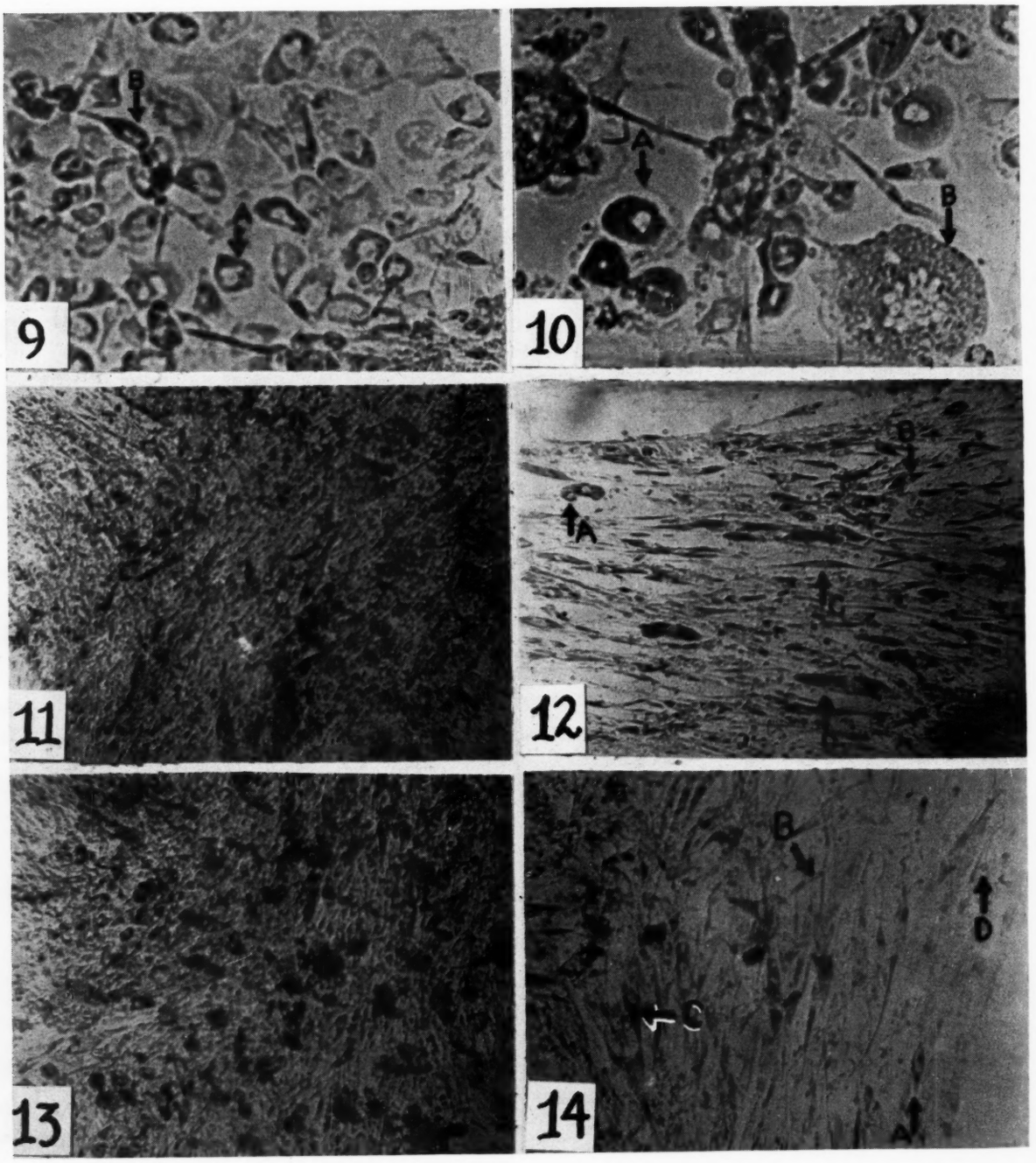
FIG. 7.—Hodgkin's lymph node, type I: 17-day-old culture in normal serum. The cell types indicated by arrows are: (*a*) the macrophage-type cell, (*b*) the reticulum cell, and (*c*) the fibroblast. Mag. ×240.

FIG. 8.—Hodgkin's lymph node, type I: the counterpart of Fig. 7, nourished with Hodgkin's serum. The difference between the two cultures (Figs. 7 and 8) is an increase in cell death as a result of the presence of Hodgkin's serum. The cell types indicated by arrows are: (*a*) the macrophage-type cell, (*b*) the reticulum cell, and (*c*) the fibroblast. Mag. ×240.

* All cultures illustrated were living and unstained when photographed.

more often, as indicated by the rate of growth of the cultures. Growth in the roller tube was allowed to continue for long periods of time (3–25 weeks) without transplantation to another tube.

Information concerning the following "diagnostic criteria" was recorded serially in the case of each culture: (*a*) the relative proportion of fixed cell and free forms, (*b*) the extent and rate of growth, (*c*) the degree of liquefaction, and (*d*) the types and individual characteristics of cells. Data relating to the presence, length of time present, number, type, size, granule or fat body content (number, size, refractility), and number of nuclei of the following cells (3) was recorded: lymphocytes (Fig. 14), fibroblasts (Figs. 4–8, 12, and 14), reticulum cells (Figs. 1, 5–9, 12, and 14), macrophages (Figs. 1, 2, 5–10), and giant cells (Figs. 5, 6, and 10).



FIGS. 9–14*

RESULTS

Hodgkin's tissues.—Alterations were observed in the morphology and cellular growth patterns of the cells nourished with most, but not all, of the Hodgkin's serum samples studied. Hodgkin's serum-induced effects of possible significance observed in 36 Hodgkin's cultures included (Figs. 5–8): greater fat granule content of the cells, larger areas of liquefaction, more free cell forms, more fat granule-containing reticulum cells, more fat granule-containing macrophage-type cells, a greater number of nuclei in the giant cells (Fig. 6), and an earlier disappearance of all types of cells and shorter life of the culture as compared with the same cells observed in normal serum cultures (Fig. 8).

Normal tissues.—The effects of Hodgkin's serum on 58 normal cell cultures considered to be significant included (Figs. 11–14): greater fat granule content of the cells (Figs. 12 and 14), larger areas of liquefaction (Figs. 12 and 14), looser fixed cell network and more irregularity of growth pattern (Figs. 12 and 14), a greater proportion of free forms (Figs. 12 and 14), more extended fibroblasts and reticulum cells (Figs. 12 and 14), more reticulum cells—both fat granule-containing and granule-free (Figs. 12 and 14), more fat granule-containing macrophage-type cells (Fig. 12), and more giant cells and a greater number of nuclei in the giant cells than in normal serum cultures.

Non-neoplastic tissues.—Hodgkin's serum effects of possible significance observed in sixteen non-neoplastic tissues obtained from patients with diseases of unknown pathogenesis included: greater fat granule content of cells, more growth, fewer macrophage-type cells, fibroblasts, and reticulum cells, and more giant cells and a greater number of nuclei in the giant cells than in normal serum cultures.

Neoplastic tissues other than Hodgkin's.—Hodgkin's serum effects of possible significance observed in eighteen neoplastic tissues other than Hodgkin's disease included (Figs. 9 and 10): greater fat granule content of the cells (Fig. 10), larger areas of liquefaction, more macrophage-type cells, both fat granule-containing and granule-free, more giant cells, and a greater number of nuclei in the giant cells than in normal serum cultures (Fig. 10).

Preliminary data indicate that sera from patients with lymphoma other than Hodgkin's disease do not induce alterations similar to the Hodgkin's serum-induced effects reported above.

The morphologic characteristics observed in the two types of Hodgkin's cellular growth (in normal serum) described in a previous paper (3) have been compared in Table 2 with the significant Hodgkin's serum-induced alterations observed in Hodgkin's, normal, non-neoplastic, and neoplastic cellular growths. Points of similarity between Hodgkin's serum-induced changes and characteristics of Hodgkin's cellular growth in normal serum appear to be the following: an increased fat granule content of cells, liquefaction of medium, number of free forms, fat granule content of reticulum cells, fat granule content of macrophage-type cells, number of giant cells, and number of nuclei in the giant cells. Alterations which were recorded only in normal cells under the influence of Hodgkin's serum were an increased number of reticulum cells and large "extended" reticulum cells. An alteration which was observed only in neoplastic

FIG. 9.—Reticulum-cell sarcoma lymph node: 7-day-old culture in normal serum. Note the granule-containing reticulum cells in the free state. The cell types indicated by arrows are: (*a*) the macrophage-type cell and (*b*) the reticulum cell. Mag ×780.

FIG. 10.—Reticulum-cell sarcoma lymph node: a parallel culture of the same tissue illustrated in Fig. 9, grown in Hodgkin's serum. The most noticeable effects are an increase in the number of giant cells and in the number of nuclei in the giant cells. The cells indicated by arrows are: (*a*) the macrophage-type cell and (*b*) the giant cell. Mag. ×780.

FIG. 11.—Normal lymph node from a newborn infant: 52-day-old culture in normal serum. Note the heavy fixed cell, network growth consisting of fibroblasts and reticulum cells containing few granules. Mag. ×240.

FIG. 12.—Normal lymph node from a newborn infant: a culture of the same 52-day-old tissue illustrated in Fig. 11, grown in normal serum 25 days and in Hodgkin's serum 27 days. The most noticeable alterations are looser sheets of fixed cells, an increase in the granule content of the cells, an increase in the number of extended reticulum cells and spindle-shaped cells, and an increase in the degree of liquefaction. The cells indicated by arrows are: (*a*) the macrophage-type cell, (*b*) the reticulum cell, (*c*) the fibroblast, and (*d*) the extended reticulum cell. Mag. ×240.

FIG. 13.—Normal thymus from a newborn infant: 52-day-old culture in normal serum. Note the heavy network composed of fibroblasts and rounded reticulum cells. Mag. ×240.

FIG. 14.—Normal thymus from a newborn infant: a culture of the same 52-day-old tissue illustrated in Fig. 13, grown in normal serum 25 days and in Hodgkin's serum 27 days. The most apparent changes are the presence of looser sheets of fixed cells, more irregularity of cellular growth, an increase in the number of extended and spindle-shaped reticulum cells, and an increase in the degree of liquefaction. The cells indicated by arrows are: (*a*) the reticulum cell, (*b*) the fibroblast, (*c*) the extended reticulum cell, and (*d*) the lymphocyte. Mag. ×240.

* All cultures illustrated were living and unstained when photographed.

cells other than Hodgkin's under the influence of Hodgkin's serum was an increased number of macrophage-type cells.

DISCUSSION

Refractile granules were seen in the cytoplasm of many of the cells observed in the cultures under the conditions described and were especially prominent in Hodgkin's type I explant growth and in normal reticulum and macrophage-type cells under the influence of Hodgkin's serum. These granules appeared in living and unstained cells as refractile bodies, were stained by Sudan IV, and ranged in size from 300 mμ to 1 μ (3).

Simms and Stillman (8) have postulated four factors in blood plasma. The "A" factor is a growth stimulant which prevents the formation of fat granules and may also dissolve them. The "B" factor, or lipfanogen, results in fat granule formation in the cells. The "C" factor is reported to produce degeneration of cells, while the "D" factor increases the cohesiveness of cells. The serum used in the experiments described in this paper by definition contained all four factors mentioned by Simms and Stillman. It is possible that one or more of these factors are decreased or increased in serum obtained from patients with Hodgkin's disease. For example, the "B" or fat granule-promoting factor (lipfanogen) may be increased in Hodgkin's serum, and the "D" or cell cohesion factor may be decreased in Hodgkin's serum.

On the other hand, the Hodgkin's serum-induced effects enumerated here may also result from (*a*) a deficiency or excess of an unknown metabolite, such as that obtained by Miller and Turner (5) from the urine of Hodgkin's patients, (*b*) a cellular deficiency of lipolytic enzyme, or (*c*) the presence of intrinsic or extrinsic macromolecular particles peculiar to the cells altered by Hodgkin's disease and arising as the result of the dissolution of these cells. The specificity of the inclusion bodies in Hodgkin's explant cells described by Grand (2) remains to be confirmed. Although it has been suggested that inclusion bodies and associated changes in normal cells brought about by the addition of supernatant fluids obtained from

TABLE 2

A Comparison of Hodgkin's Serum-induced Morphologic and Growth Pattern Alterations with the Characteristics of Hodgkin's Tissue Growth Types I, Ia and II in Normal Serum

Effect of Hodgkin's serum: A Hodgkin's Cells	B Normal Cells*	C Non-neoplastic Cells**	D Neoplastic Cells***	Alteration in morphology or cellular growth pattern not observed in control cultures. Increased:	Summary of Hodgkin's sera effects on normal and diseased cells. (see columns A-D)	Characteristics of Hodgkin's tissue growth types I, Ia, II. (normal serum)
±	±	±	±	1. Growth	±	±
++	++	+	++	2. Granule-content (fat bodies) of cells	+	+
+	++	0	+	3. Liquefaction of medium	+	+
+	+	0	+	4. Free cell forms	+	+
+	0	0	0	5. Cell death	+ (in A)	±
±	±	±	±	6. Lymphocytes	±	±
0	0	0	+	7. Macrophage-type cells	+ (in D)	+
0	+	0	0	8. Reticulum cells	+ (in B)	+
0	+	0	0	9. Large "extended" reticulum cells	+ (in B)	+
+	+	0	0	10. Granule content (fat bodies) of reticulum cells	+	+
+	+	0	+	11. Granule content (fat bodies) of macrophage-type cells	+	+
0	+	+	++	12. Giant cells	+	+
+	+	+	++	13. Nuclei in giant cells	+	+

Legend: 0 = absence of alteration; ± = variable; + = presence of alteration; ++ = alteration pronounced; — = comparison; °°° = comparison in some cases; ---- = comparison variable

* = neonatal and adult tissue cells.
** = non-neoplastic diseases of unknown etiology and non-specific lymphadenitis tissue cells.
*** = neoplastic diseases including lymphoblastoma other than Hodgkin's disease.

Hodgkin's cells in tissue culture are the result of the presence of a virus (2), it is possible that these changes may be metabolic in origin. It is to be expected that the Hodgkin's cell growing *in vivo* and *in vitro* gives rise to one or more abnormal metabolites which may effect the growth of both normal and abnormal inoculated cells in tissue culture.

The serum-induced alterations described in the normal and abnormal cells studied appear to bear a qualitative resemblance to the fundamental alterations which characterize the growth of Hodgkin's explant cells in tissue culture. The significance of this observation cannot be judged until further study clarifies the nature and establishes the specificity of these changes.

Although sera obtained from lymphomata other than Hodgkin's disease do not produce the morphology and growth pattern changes described in the case of Hodgkin's serum, it is not justifiable to conclude that these serum-induced alterations are specific and limited to Hodgkin's sera until abnormal sera of many varieties have been studied under the same conditions.

SUMMARY

1. A study of the effect of normal human serum and Hodgkin's serum on normal and diseased human cells in tissue culture is described.

2. The potentially significant Hodgkin's serum-induced alterations which have been noted are: (*a*) an increased fat granule content of reticulum and macrophage-type cells, (*b*) an increase in liquefaction, (*c*) an increase in free cell forms, (*d*) an increase in the number of giant cells in all tissues with the exception of Hodgkin's, (*e*) an increase in the number of giant cell nuclei in Hodgkin's and other explant cells studied, and (*f*) a decreased life span of Hodgkin's cells, but not of other cells. More pronounced and more varied individual alterations are observed in normal neonatal cells under the influence of Hodgkin's serum than in diseased cells of the types observed.

3. The Hodgkin's serum-induced morphologic changes in normal and diseased cells appear to bear a qualitative resemblance to the fundamental alterations which characterize the growth of Hodgkin's explant cells in the presence of normal serum in tissue culture.

4. Although the serum-induced alterations in Hodgkin's disease appear to be specific within the area of the lymphomata, it is not justifiable to conclude that they may not be duplicated by the serum of one or more other diseases. Further investigation will reveal the degree of specificity of the phenomena observed.

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The Virus-induced Rabbit Papilloma-to-Carcinoma Sequence

III. Immunological Tests for Papilloma Virus in Cottontail Carcinomas*

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Benign papillomas on wild cottontail rabbits (*Sylvilagus floridanus alacer* Bangs) from Iowa and Kansas were found by Shope (7) to yield a virus readily transmissible to domestic rabbits (7) and by Shope and others to several species of wild hares (see footnote 4 in reference 13). A marked difference in host reactivity became apparent early, as shown by the easy recoverability of the virus from the tumors on hares, in contrast to the more usual lack of, or at best limited, recoverability of the virus from domestic rabbits. This difference in host reactivity is without apparent directive influence on the ultimate fate of the benign tumors, for both natural and experimental growths on various host species undergo successive changes to regression or to cancer (12). The relationship of the virus to the ultimate cancer, moreover, continues to be unknown. All attempts to recover the virus directly from cancers on any host have failed, and the indirect evidence for its presence, which derives from two domestic rabbits, leaves open the question of the continued presence of the virus during the papilloma-to-carcinoma sequence.

Kidd, Beard, and Rous (4) reported in 1936 that a single animal of the 17 domestic rabbits that had been utilized as recipients for the transplantation of metastatic cancerous tissue showed progressive growth of the transplant and that serum from this rabbit neutralized not more than 20 units of virus (this approximates about a tenfold variation in titer). "Its titer was low."

* This investigation was aided by a grant from the Jane Coffin Child's Memorial Fund for Medical Research.

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Received for publication February 27, 1950.

Kidd and Rous (5) later described a second cancer from a domestic rabbit which over several years they have successfully transplanted through many generations. Transplantation of this cancer in series was effected with equal success in normal and in papilloma-immune animals (3). When the tumor was returned to normal domestic rabbits after serial passage through hyperimmunized hosts, these yielded serum containing specific antibodies for papilloma virus. These facts were interpreted to mean that papilloma virus was carried for many months in "masked" or altered form as an "enduring partnership." It was a remarkable example of the capacity of viruses to survive in tumor cells as inapparent passengers. Obviously, however, the essential question, the exact relationship of papilloma virus to the cancer, remained open.

We attempted early to learn whether the virus could be demonstrated by direct or indirect methods in the cancers that followed both naturally and experimentally induced papillomas (8,13). Because the cottontail rabbit, as the natural host for the virus, might well be the best laboratory animal for the investigation of the relationship of the virus to the successive alterative changes that occur over many months, we approached the problem through the use of carcinomatous material derived from cottontail rabbits. If virus were present in such cancers, it should be readily demonstrable, for it is customary to recover virus from papillomas on this host.

The plan for our experiments was: (*a*) to introduce cancerous tissue from western into eastern cottontail rabbits; (*b*) if overt lesions did not appear, to test these host animals for acquired immunity to papilloma virus, directly by measuring their resistance to re-infection and indirectly by assessing their serum for antibodies to papilloma virus. The carcinomatous tissue was to be used in

the form of primary lesions, metastases, and after irradiation with 2,000,000 roentgen units of x-ray.[1]

It is the purpose of the present paper, the third in a series, to record the results of immunological studies designed to throw light on the role of Shope's papilloma virus in the papilloma-to-carcinoma sequence.

EXPERIMENTAL

The same experimental plan was followed for the first three experiments. For purposes of orientation, therefore, the first and simplest is described in detail.

Cottontail rabbit S12-74, which died accidentally 342 days after its arrival from Kansas, provided the carcinomatous tissue. It had six papillomas on arrival. During the course of the next several months three of these tumors regressed and disappeared. Of the remaining, microscopic examination established one as a benign papilloma and two as cancers. A single right axillary node was largely replaced by cancer.

The cancerous tissue was mixed and triturated in Locke's solution to yield a 10 per cent suspension. Each of eight cottontails was injected with 0.1 ml. of this suspension at each of four sites, two subcutaneously and two intraperitoneally, and two domestic rabbits were inoculated by cross-hatch scarification, that we might learn by direct tests whether or not virus was present in the benign papilloma or in the cancers.

Forty-five days later, when no reaction was noted, except a slight pyogenic infection at several sites, each of these rabbits was tested for evidence of acquired immunity. These tests fell into two categories: (*a*) tests for active immunity in which the recipients were inoculated by cross-hatch scarification with virus in tenfold decrements, to learn whether some evidence for resistance could

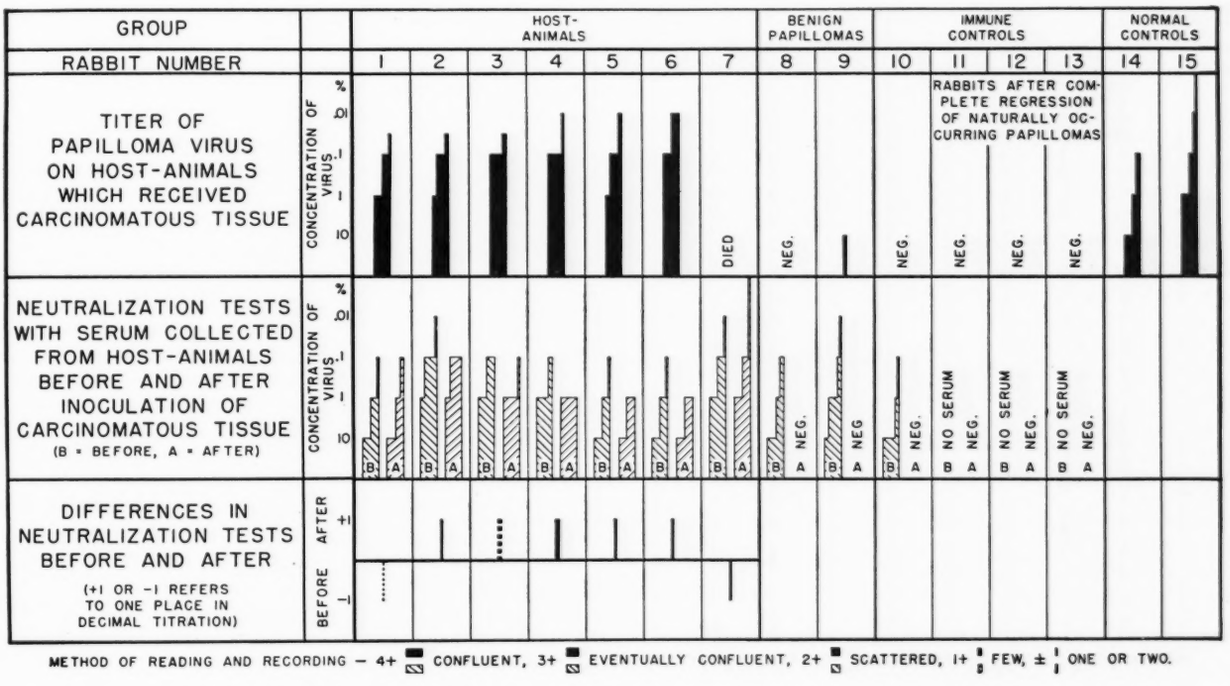

EXPERIMENT I
TESTS TO DETERMINE
ACTIVE AND PASSIVE IMMUNITY OF HOST-ANIMALS
TO PAPILLOMA VIRUS
GROUP
HOST-ANIMALS
BENIGN PAPILLOMAS
IMMUNE CONTROLS
NORMAL CONTROLS
RABBIT NUMBER
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
TITER OF PAPILLOMA VIRUS ON HOST-ANIMALS WHICH RECEIVED CARCINOMATOUS TISSUE
CONCENTRATION OF VIRUS
RABBITS AFTER COMPLETE REGRESSION OF NATURALLY OCCURRING PAPILLOMAS
DIED
NEG.
NEUTRALIZATION TESTS WITH SERUM COLLECTED FROM HOST-ANIMALS BEFORE AND AFTER INOCULATION OF CARCINOMATOUS TISSUE
(B = BEFORE, A = AFTER)
NO SERUM
DIFFERENCES IN NEUTRALIZATION TESTS BEFORE AND AFTER
(+1 OR −1 REFERS TO ONE PLACE IN DECIMAL TITRATION)
AFTER
BEFORE
METHOD OF READING AND RECORDING — 4+ CONFLUENT, 3+ EVENTUALLY CONFLUENT, 2+ SCATTERED, 1+ FEW, ± ONE OR TWO.

FIG. 1.—Summary of the data for the tests employed in Experiment 1 to determine whether the inoculation of cottontail carcinomatous tissue into cottontail rabbits had resulted in specific anti-viral effects.

In Figures 1, 2, 3, and 5 the reaction to inoculation of papilloma virus in 10-fold decrements has been charted in such a way that the width of the column indicates the degree of the reaction: entire width equals confluent or 4+ reaction; thin line equals 1 or 2 papillomas or ± reaction.

[1] Because we knew from previous experience (11, 14) that 2,000,000 roentgen units kill all tissue cells and bacteria but are without detectable effect on papilloma virus, we employed this enormous dose of x-ray in the hope that it might free "masked" virus (1, 2, 5), if such were present, in the cancerous tissue.

be elicited, and (*b*) tests for passive immunity in which a sample of serum withdrawn 30 days after the injection of cancerous tissue was tested for its ability to neutralize papilloma virus. These latter tests were carried out by measuring the neutralizing effect of equal parts of undiluted serum on the virus when it was present in successive concentrations that represented tenfold decrements. As a control for each sample of serum under test, a sample which had been withdrawn from the same animal before injection was tested concurrently and in duplicate on normal domestic rabbits.

Experiment 1.—The results of Experiment 1 are presented in Figure 1.

In this pilot experiment only 15 animals were used: 7 host animals, which received carcinomatous material, and 8 controls. Their reaction to the virus is charted in such a way that the width of the column indicated the degree of the reaction—from confluent, 4 + (6), to 1 or 2 papillomas, a ± reaction. As an example, note the two normal animals, Nos. 14 and 15, included to establish the titer of the sample of virus used. With No. 15, a 1–10 dilution gave a confluent or 4+ reaction, a 1–100,000 dilution gave only a single papilloma, a ± reaction. It can be seen in Figure 1, furthermore, that the host animals, Nos. 1–6, when tested for active immunity against papilloma virus (tested by the injection of successive tenfold decrements of the virus), reacted in a way essentially similar to that of the normal animals, Nos. 14 and 15. This finding is in striking contrast to the reaction of the control cottontails, Nos. 10–13, which had a high degree of immunity as the result of having carried papillomas.

The results of the tests for passive immunity are presented in the second division of Figure 1. Sera collected from the "host animals" before, as indicated by *B*, and 30 days after the inoculation of carcinomatous tissue, as indicated by *A*, were mixed with graded doses of virus (the final concentration of virus is shown on the chart) and tested on normal animals. The results, as shown by the comparative widths of each pair of columns, indicate no striking difference between the neutralizing ability of serum withdrawn from rabbit 30 days after the inoculation of carcinomatous tissue and that withdrawn before. The results of these tests for passive immunity thereby substantiate the tests for active immunity, in which the reaction to injection of virus was essentially that of normal animals. To ascertain whether the differences were significantly different, however, the observed differences in end-point are charted in the third division of Figure 1. It might seem at first view that the sera withdrawn after inoculation with carcinomatous tissue had greater neutralizing ability than that withdrawn before, but these differences reflect the small number of sera tested, as will become apparent in succeeding experiments.

When the results of the first experiment yielded no evidence for the presence of papilloma virus in carcinomatous tissue from cottontail rabbits, the experimental approach was altered for the second experiment to include a larger number of rabbits and to carry out each titration in duplicate.

Experiment 2.—Cottontail rabbit S16-04 had two large papillomas on its arrival from Kansas. When this animal died 300 days later, cancers had replaced both papillomas, and metastatic cancers were present in a left inguinal lymph node, a right axillary lymph node, and in the lungs.

The cancerous tissue was made ready for inoculation by cutting it into pieces of such size that, suspended in Locke's solution, they readily passed the lumen of a 14-gauge needle. Four preparations were employed for inoculation: preparations 1 and 2 were made from a mixture of both primary cancers; preparations 3 and 4 were prepared from a mixture of the metastatic cancers. For inoculation, preparations 1 and 3 were injected without further treatment, whereas preparations 2 and 4 were irradiated with 2,000,000 r (11) immediately before injection. The injections were made intramuscularly at two sites, subcutaneously at two sites, cross-hatch scarification at two sites, intraperitoneally, and tissue fragments were placed in the anterior chamber of the eye of four rabbits. Forty-four eastern cottontails (Nos. 16–59) were used as recipients. Each had been bled for control serum.

The sites of inoculation on each animal were examined at weekly intervals. When 45 days had elapsed without evidence for growth of the transplanted tumor tissue in a single animal, each of the 44 cottontails was bled for test serum. These test animals and four normal eastern cottontails were then assessed for active immunity to papilloma virus by duplicate inoculations by cross-hatch scarification of papilloma virus in decimal dilutions. Further tests to determine whether the injection of the cancerous fragments had resulted in the formation of specific neutralizing antibodies for papilloma virus were effected by measuring concurrently the antibody content of the samples of serum collected from each animal before and after its inoculation with carcinomatous tissue.

In Figure 2 are summarized the composite results of Experiment 2. These results are presented in the same form as described for Figure 1.

It can be seen in Figure 2 that 38 of the 44 host animals survived the second bleeding to be tested for active immunity to papilloma virus. The reaction of each of these 38 rabbits (Nos. 16–53) to inoculation with successive decimal dilutions of papilloma virus was essentially similar to that of each of the four normal controls (Nos. 60–63).

It can be seen further, moreover, that the injection of carcinomatous tissue did not significantly alter in any host animal the neutralizing capacity of its serum, i.e., a graded amount of virus elicited papillomas which were essentially the same as to size and incubation period, whether it had been mixed with serum from the sample withdrawn before inoculation with carcinomatous tissue or with serum withdrawn after its injection. The variations in end-point, which reflect the usual biological variation, are depicted as "differences in neutralization tests before and after" in the third horizontal subdivision of Figure 2.

It is clear that Experiment 2 failed to yield evidence for the presence of papilloma virus in the carcinomatous tissue. Indeed, through utilization of a large number of rabbits and by carrying out each titration in duplicate, it might seem that these results would suffice. When a cottontail rabbit that harbored at the time of its death from intercurrent infection primary cancers, metastatic cancers, and a benign papilloma became available,

EXPERIMENT II

TESTS TO DETERMINE ACTIVE AND PASSIVE IMMUNITY OF HOST-ANIMALS TO PAPILLOMA VIRUS

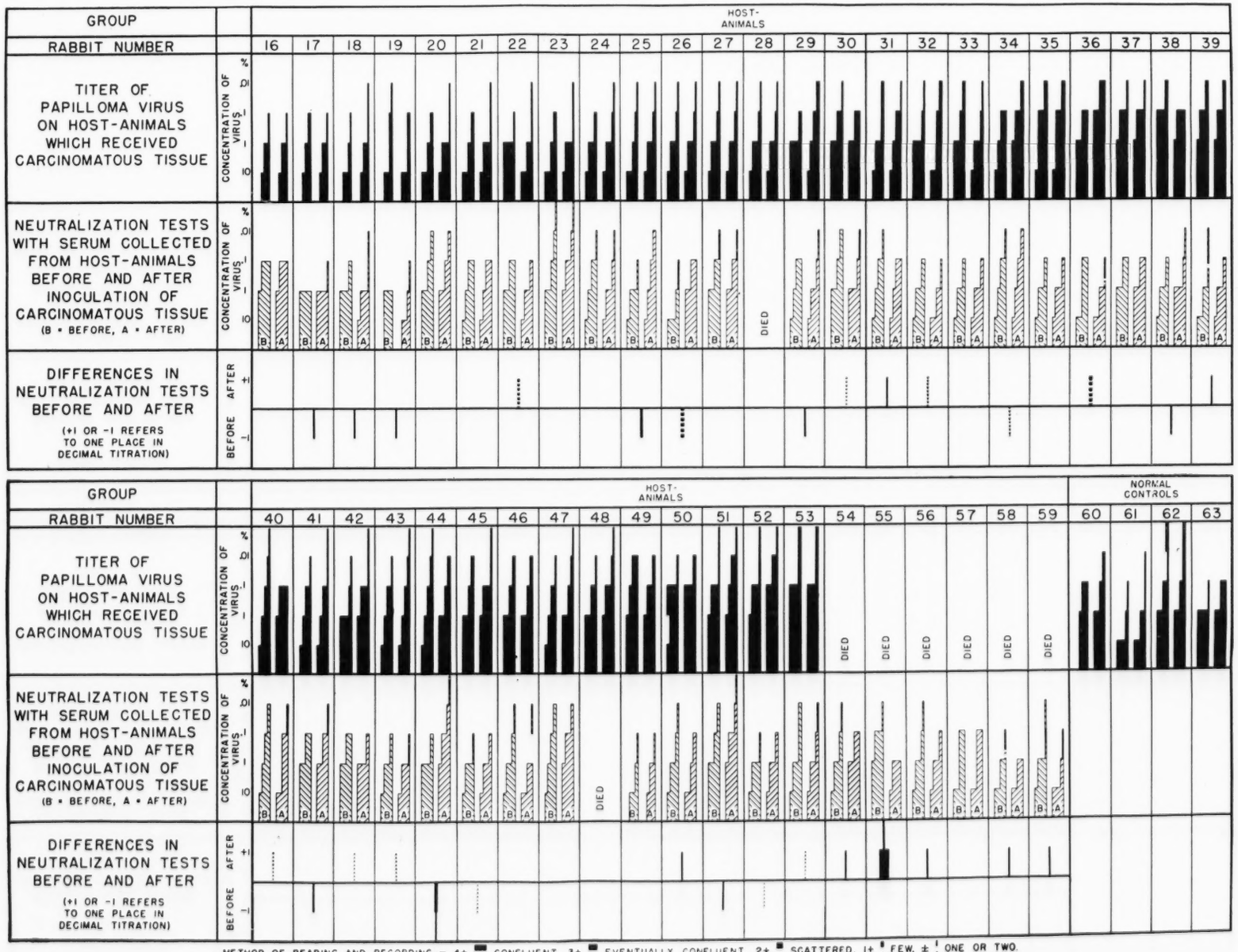

GROUP
HOST-ANIMALS
RABBIT NUMBER
16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39
TITER OF PAPILLOMA VIRUS ON HOST-ANIMALS WHICH RECEIVED CARCINOMATOUS TISSUE
CONCENTRATION OF VIRUS
NEUTRALIZATION TESTS WITH SERUM COLLECTED FROM HOST-ANIMALS BEFORE AND AFTER INOCULATION OF CARCINOMATOUS TISSUE
(B = BEFORE, A = AFTER)
DIED
DIFFERENCES IN NEUTRALIZATION TESTS BEFORE AND AFTER
(+1 OR -1 REFERS TO ONE PLACE IN DECIMAL TITRATION)
AFTER
BEFORE
GROUP
HOST-ANIMALS
NORMAL CONTROLS
RABBIT NUMBER
40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63
TITER OF PAPILLOMA VIRUS ON HOST-ANIMALS WHICH RECEIVED CARCINOMATOUS TISSUE
NEUTRALIZATION TESTS WITH SERUM COLLECTED FROM HOST-ANIMALS BEFORE AND AFTER INOCULATION OF CARCINOMATOUS TISSUE
(B = BEFORE, A = AFTER)
DIFFERENCES IN NEUTRALIZATION TESTS BEFORE AND AFTER
(+1 OR -1 REFERS TO ONE PLACE IN DECIMAL TITRATION)
METHOD OF READING AND RECORDING - 4+ CONFLUENT, 3+ EVENTUALLY CONFLUENT, 2+ SCATTERED, 1+ FEW, ± ONE OR TWO.

FIG. 2.—Summary of the data for the tests employed in Experiment 2 to determine whether the inoculation of cottontail carcinomatous tissue into cottontail rabbits had resulted in specific papilloma anti-viral effects.

EXPERIMENT III

TESTS TO DETERMINE ACTIVE AND PASSIVE IMMUNITY OF HOST-ANIMALS TO PAPILLOMA VIRUS

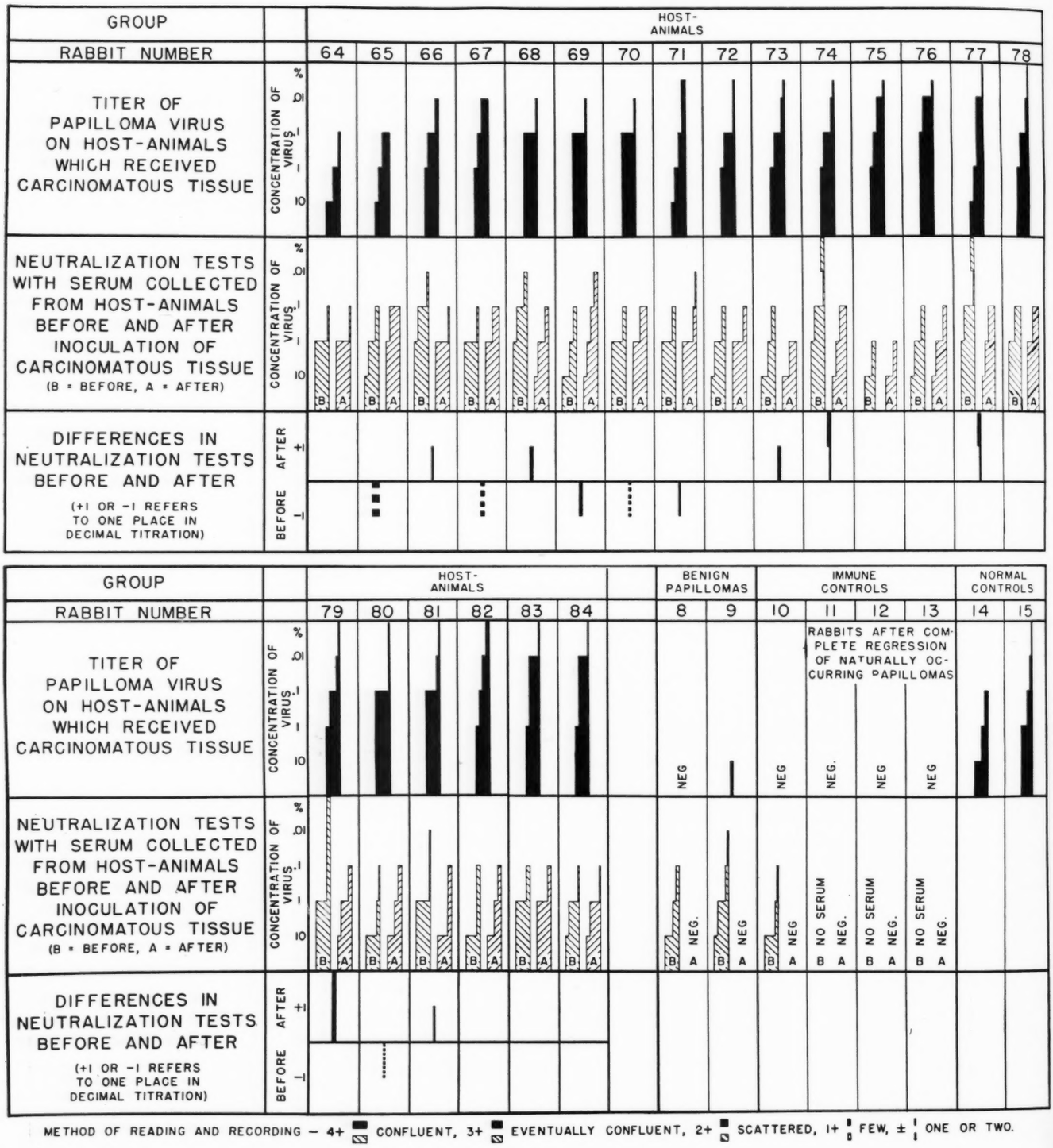

GROUP
HOST-ANIMALS
RABBIT NUMBER
64 65 66 67 68 69 70 71 72 73 74 75 76 77 78
TITER OF PAPILLOMA VIRUS ON HOST-ANIMALS WHICH RECEIVED CARCINOMATOUS TISSUE
CONCENTRATION OF VIRUS
%
10
1
.1
.01
NEUTRALIZATION TESTS WITH SERUM COLLECTED FROM HOST-ANIMALS BEFORE AND AFTER INOCULATION OF CARCINOMATOUS TISSUE
(B = BEFORE, A = AFTER)
B A
DIFFERENCES IN NEUTRALIZATION TESTS BEFORE AND AFTER
(+1 OR -1 REFERS TO ONE PLACE IN DECIMAL TITRATION)
AFTER
BEFORE
+1
-1
GROUP
HOST-ANIMALS
BENIGN PAPILLOMAS
IMMUNE CONTROLS
NORMAL CONTROLS
RABBIT NUMBER
79 80 81 82 83 84 8 9 10 11 12 13 14 15
RABBITS AFTER COMPLETE REGRESSION OF NATURALLY OCCURRING PAPILLOMAS
NEG
NEG.
NO SERUM
METHOD OF READING AND RECORDING — 4+ CONFLUENT, 3+ EVENTUALLY CONFLUENT, 2+ SCATTERED, 1+ FEW, ± ONE OR TWO.

FIG. 3.—Summary of the data for the tests employed in Experiment 3 to determine whether the inoculation of cottontail carcinomatous tissue into cottontail rabbits had resulted in specific papilloma anti-viral effects.

it was, nevertheless, decided to try a third experiment.

Experiment 3.—The carcinomatous tissue was removed from cottontail rabbit S12-76, which, on arrival from Kansas, had eighteen papillomas. When the animal died 229 days later, seven papillomas had regressed, nine papillomas had become confluent to yield five cancers, two papillomas still were present, and three axillary nodes were replaced by cancer. By using for direct transfer by cross-hatch inoculation an inoculum prepared from each of the fifteen lesions, papillomas resulted from a single tumor, a benign-appearing papilloma removed from over the lower back. The method of preparation of the tissue suspensions and the technic for transfer to the recipient rabbits were the same as for Experiment 2. The results are presented in Figure 3.

elicited when four cottontails (Nos. 10–13) were tested after their naturally occurring papillomas had regressed.

One point needs special emphasis. The cottontail from which the carcinomatous material was derived had at the time of its death primary cancers at four sites, metastatic cancers in three lymph nodes, and a single benign papilloma. When the benign tumor was tested for virus on Nos. 8 and 9, it yielded confluent papillomas, and these hosts, as depicted in Figure 3, developed a high degree of active and passive immunity. In striking contrast to these results, not one of the seven cancers coming from the same cottontail yielded evidence for the presence of papilloma virus when the respective hosts were tested for active and passive immunity.

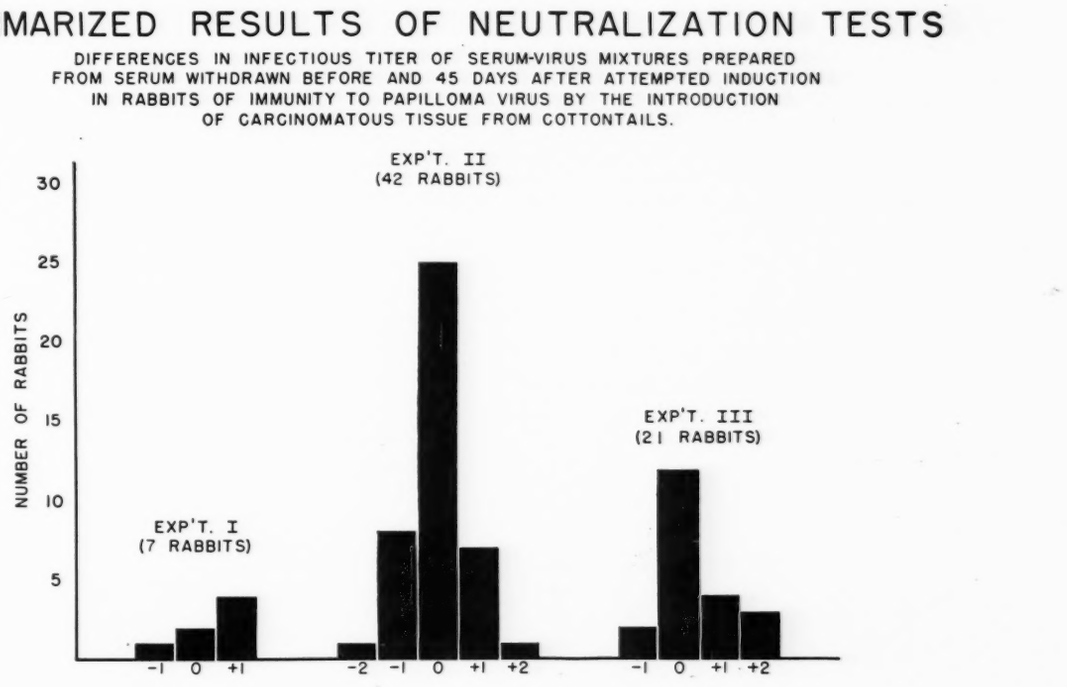


FIG. 4.—A summary for Experiments 1, 2, and 3 of the differences in infectious titer of serum-virus mixtures prepared from serum withdrawn before and 45 days after attempted induction in cottontail rabbits of immunity to papilloma virus by the introduction of carcinomatous tissue from cottontail rabbits.

+ = neutralization greater after than before injection of carcinomatous tissue.

− = neutralization greater before than after injection of carcinomatous tissue.

0 = neutralization same before and after injection of carcinomatous tissue.

1 = 10-fold difference in final titer.

2 = 100-fold difference in final titer.

It is clear that the data in Figure 3 substantiate the findings already described for the preceding experiments. The 21 host animals, Nos. 64-84, were without visible or palpable reaction to the inoculation of carcinomatous tissues. Moreover, none showed evidence of antibody formation when tested directly for actively acquired immunity, or when tested indirectly by assessing its serum for neutralizing antibodies for papilloma virus. The reaction to the inoculation of papilloma virus was similar to that of normal control animals, Nos.14 and 15, and totally different from the reactions

The differences in neutralizing ability for the samples of serum withdrawn before the inoculation of carcinomatous material and the samples of serum withdrawn after the inoculation of carcinomatous material have been depicted in the third horizontal column of each figure.

It has been pointed out, in presenting the results of each of the three experiments, that there were

no significant differences. The variations from animal to animal, or on the same animal, are merely the expected variation about the biological endpoint. These data are summarized in Figure 4.

In Experiment 1 a small number of rabbits was used. Experiment 2 was designed to include a large number of animals. Neutralization was the same for the majority of animals. A few sera gave greater neutralization after than before the inoculation of carcinomatous tissue; a few sera the opposite, i.e., greater neutralization before than after. The composite results, as presented in Figure 4, give a good distribution curve. It can be seen in Figure 4, moreover, that the results of Experiment 3 substantiate the findings of the preceding experiments.

Even though the results of these experiments did not support the hypothesis that the cancers had papilloma virus in their cells, a fourth experiment was designed to test whether the parenteral introduction of massive doses of carcinomatous tissue would give evidence for the presence of papilloma virus.

Experiment 4.—Metastatic carcinomatous tissue and papillomatous tissue, as mixtures derived from several cottontail rabbits, were utilized for testing. Each mixture was triturated with alundum in Locke's solution to yield 10 per cent suspensions of tissue, centrifuged to remove the larger particles and irradiated with 2,240,000 r to free it from extraneous bacterial contaminants. Twelve cottontail rabbits were used as recipients for these irradiated mixtures. Six of them (Nos. 85–90) were given four successive intraperitoneal doses of 4 ml. of the carcinomatous tissue at 7-day intervals. One (No. 90) died of intercurrent infection. Into the other 6 (Nos. 91–96) an equal amount of papillomatous tissue was injected for control purposes.

When these eleven survivors, after 45 days' observation, had shown no effects from the injections, each was tested for evidence of active and passive immunity to papilloma virus. The data for these tests and for additional control tests are summarized in Figure 5. It can be noted that to measure better the comparable reactivity of the two test groups in the immunity tests, three additional groups of cottontail rabbits were included: normal animals that served to establish the infectious titer of the challenge dose of virus; rabbits with pre-existing papillomas; rabbits with known carcinomas. The two latter groups, therefore, comprised animals exhibiting a high grade of naturally acquired immunity.

It is evident from the figures that tests for active immunity, which were carried out in duplicate on the recipients of carcinomatous tissue and in triplicate on normal animals, yielded similar results. Indeed, multiple titrations on a single animal showed no greater variation than the titrations on different animals. In marked contrast to these results, the recipients of the papillomatous tissue (Nos. 91–96) developed a high grade of active immunity, i.e., virus known to have an infectious titer of 10^5 elicited two small papillomas on but one of the six animals tested. Thus, their reactions were entirely within the range of reactivity which was shown by the animals in the two groups of immune controls (Nos. 97–102).

The tests for passive immunity in each group reflected the same immune state for each animal. It can be seen in Figure 5 that the serum samples which were without neutralizing effect on papilloma virus had been withdrawn from the host animals (recipients of the carcinomatous tissue, Nos. 85–89) both before and after the injection of cancerous tissue, but from the controls (recipients of the papillomatous tissue, Nos. 91–96) before the injection of papillomatous tissue only. The samples of serum withdrawn from the rabbits in the latter group after the injection of papillomatous tissue, on the other hand, contained viral antibodies adequate to neutralize 10,000 infectious doses of papilloma virus. In fact, the neutralizing ability of each of the samples of serum in this latter group was at least as great as any of the samples derived from rabbits with long-existing papillomas (Nos. 97–99) or carcinomas (Nos. 100–102).

DISCUSSION

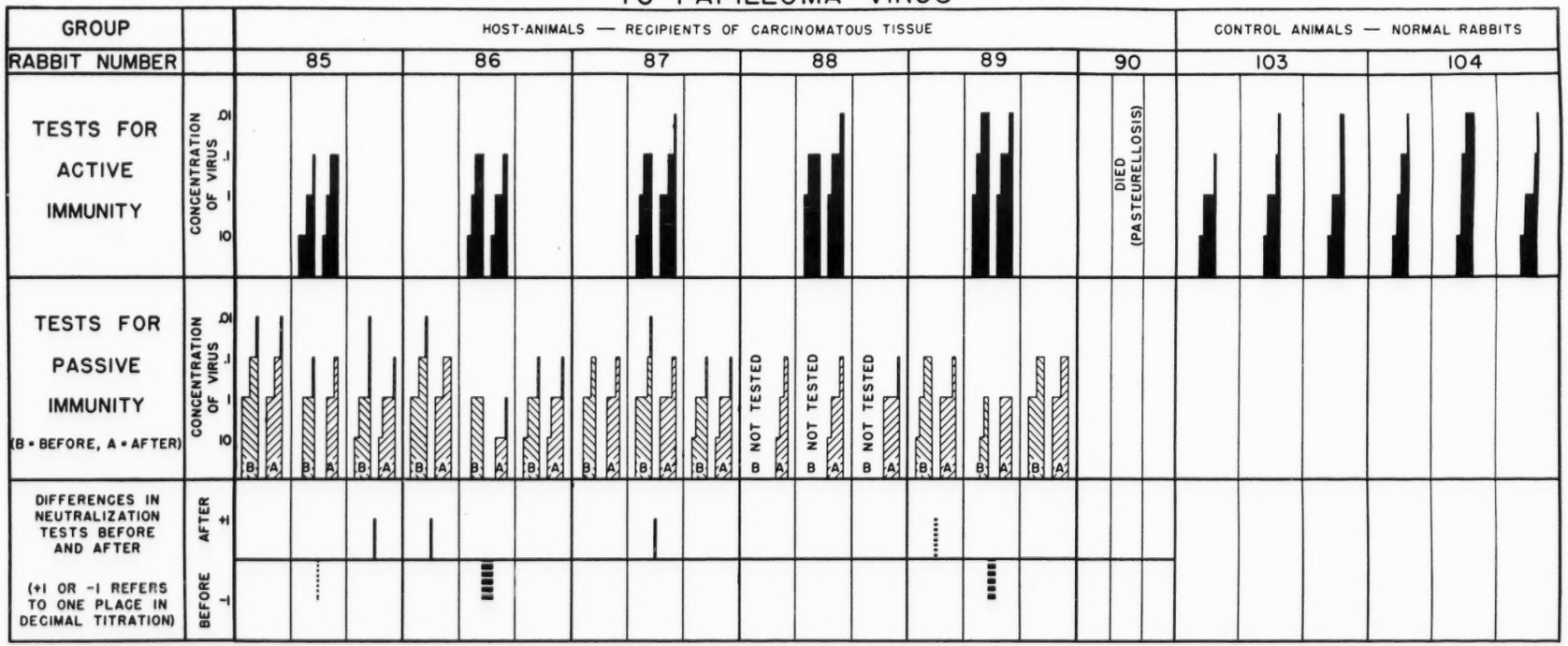
Under the experimental conditions described in the present paper, it was found that carcinomatous tissue from wild cottontail rabbits yielded no evidence for the presence of papilloma virus. When naturally occurring virus-induced papillomas on cottontails were permitted to develop without outside interference through successive stages in their developmental cycle to become epidermoid carcinomas, transfer of such carcinomatous tissue to normal eastern cottontail rabbits failed regularly to elicit visible or palpable evidence of tumor growth or immunologic evidence for the presence of papilloma virus in the carcinomatous tissue. Both primary cancers and metastatic cancers in suspension were employed for inoculation by the subcutaneous, intramuscular, intraperitoneal, intraocular and cutaneous routes, both directly and after irradiation with an amount of x-ray lethal for bacterial contaminants and tissue cells but without effect on papilloma virus (11).

Eastern cottontail rabbits were employed as the recipient test animals because it had been established (9,12) for cottontails captured in New York that: (*a*) they were fully susceptible to infection by the strains of papilloma virus that had been obtained from western cottontail rabbits, (*b*) infection by the virus resulted in tumors indistinguishable in their pattern of growth (12) from the naturally induced papillomas on their western cousins, and (*c*) their papillomas, when in the proliferative or early stationary phases, readily yielded the virus in high titer. The utilization of this host would, it would seem, have been optimal

EXPERIMENT IV
TESTS TO DETERMINE

EXPERIMENT IV

TESTS TO DETERMINE ACTIVE AND PASSIVE IMMUNITY OF HOST-ANIMALS TO PAPILLOMA VIRUS



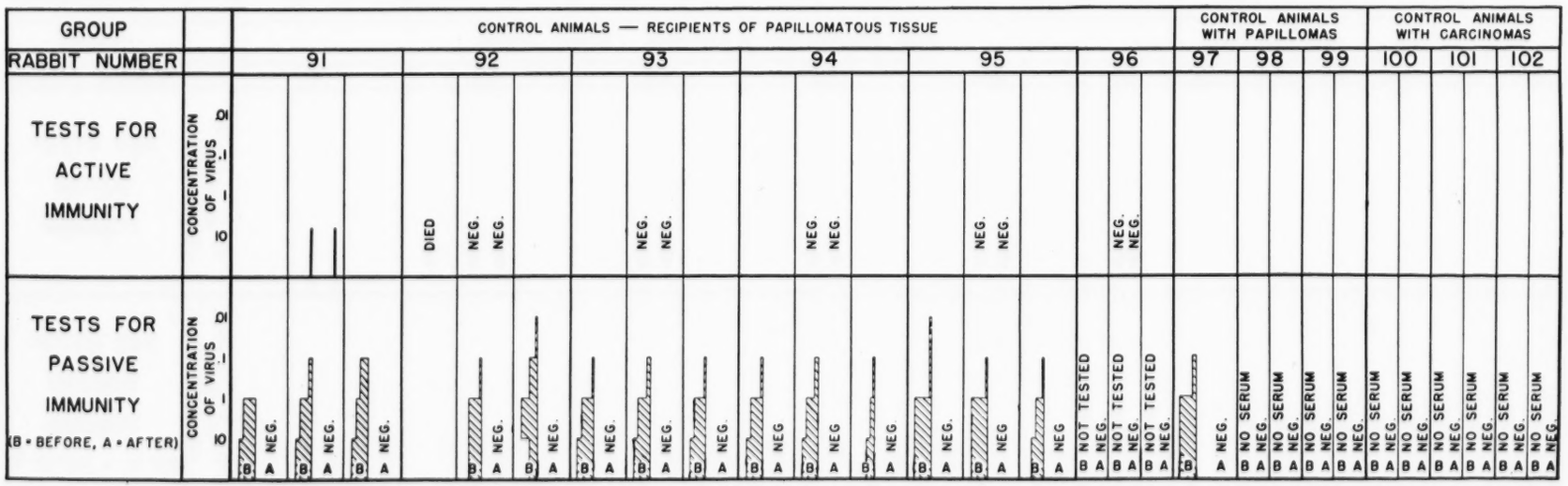


FIG. 5.—Summary of the data for the tests employed in Experiment 5 to determine whether the inoculation of cottontail carcinomatous tissue into cottontail rabbits had resulted in specific papilloma anti-viral effects.

for the recovery of the virus, had it been present in the cancers.

The results of this immunological study support the hypothesis that the role of papilloma virus in the rabbit papilloma-to-carcinoma sequence is limited to that of a provocative carcinogenic agent and not to that of an activating carcinogen. Indeed, the early disappearance of papilloma virus from the papillomas of domestic rabbits, and the absence of the virus during the stationary and involutionary growth phases of papillomas on domestic and cottontail rabbits (12) make our failure to demonstrate virus in naturally occurring cancers predictable. The results of the present study, also, substantiate our earlier findings (8,13) in which the lesions from 26 of 33 wild cottontail rabbits with epidermoid carcinomas, established at autopsy, were tested for the presence of papilloma virus. We found that 12 of 40 histologically benign papillomas yielded the virus, but none of 106 cancers did.

It would be most surprising if papilloma virus were not demonstrated in an occasional cancer (4, 5). This would not mean that the virus played an etiological role. Its presence might be explained in a number of ways: by a direct carry-over of papilloma cells into the cancer; by cancer cells carrying papilloma virus as a "passenger." It is established that these cancer cells can be infected by nonetiologically significant viruses (10). Our consistent failure to demonstrate papilloma virus in the cancers of cottontail rabbits, therefore, makes it seem improbable to us that papilloma virus forms an essential part of the ultimate cancer.

SUMMARY

Studies were designed to elucidate the relationship of Shope's papilloma virus to the carcinoma that often develops when rabbits have long carried virus-induced papillomas. Carcinomatous materials from western cottontail rabbits were tested for the presence of virus by utilizing eastern cottontails as test animals, because it is the rule to recover papilloma virus from papillomas on this species of host. There were available for study 106 cancers removed from 26 cottontails. For a single experiment mixed carcinomatous material from many hosts was employed, and for each of three experiments the carcinomatous material utilized came from single animals.

It was found that the transfer of carcinomatous material to normal animals did not result in an increased resistance to papilloma virus, nor did serums from these animals acquire neutralizing capacity for papilloma virus. One of the cottontails provided eight lesions for investigation: primary cancers at four sites, metastatic cancers in three lymph nodes, and a single benign papilloma. Papilloma virus was recovered from the papilloma, and a high degree of immunity resulted in the recipient test animals that received tissue, but none of the cancers from the same animal yielded evidence for the presence of papilloma virus.

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The Significance of the Anterior Chamber in Tumor Transplantation

I. Transplantation of Mouse Neuroblastoma C1300 to Homologous Hosts*†

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Greene (1) has stated that, in transplantation of mouse tumors into nonsusceptible strains, "the so-called influence of strains appears to be largely negated when the anterior chamber is used."

This investigation is an attempt (*a*) to verify this observation and (*b*) to determine whether or not growth of a tumor transplanted to the anterior chamber is limited to this space or progresses beyond it, in a host in which the same tumor would not grow if transplanted subcutaneously.

MATERIALS AND METHODS

The mouse neuroblastoma C1300 and the C57 brown strain of mice were selected, because subcutaneous transplants of this tumor grow progressively in only a small percentage of mice of this strain. The tumor arose spontaneously in strain A mice and was obtained from Bar Harbor, Maine. The C57 brown mice came from the same source. Mice of the highly susceptible ABC strain (Bar Harbor) served as control animals.

Three hundred and twenty-seven female C57 brown mice weighing about 15 gm. were inoculated with C1300; 161 mice received subcutaneous grafts and 166 received grafts into the anterior chamber. Both groups were anesthetized with sodium nembutal. The inoculations in both sites were performed with a gauge # 20 spinal needle. The fragments were of similar size, taken from the same tumor, and inoculated simultaneously. The subcutaneous grafts were placed in the midline halfway between the symphysis pubis and the ensiform process. The anterior chamber grafts were inoculated under a stereoscopic microscope after the corneae had been cut with a pointed Parker blade.

Eight to ten mice of each group were killed 6 hours after inoculation, 24 hours after inoculation, on the second, third, fourth, fifth, sixth, eighth, tenth, twelfth, fifteenth, nineteenth, and twenty-fourth day. The remaining animals were not killed.

At the time of sacrifice, the eyes and adjacent structures were removed and cut sagittally. In the subcutaneously grafted animals the abdominal wall was reflected; when the graft was visible through the muscle layer, it was excised with the skin and muscle covering. When it was not visible, the muscle was stripped under the stereoscopic microscope; both muscle and subcutaneous surfaces were searched and suspected areas removed for sectioning. The tissues were fixed in formalin; between 5 and 40 sections were prepared from each, stained with hematoxylin and eosin, and evaluated with regard to the presence or absence of tumor growth. Grafts were considered as growing when there was mitotic activity of tumor cells or vascularization of viable tumor tissue.

In some subcutaneously inoculated mice, repeat sections failed to show the graft site. Whenever this occurred in mice sacrificed during the first week after inoculation, it was considered to be possibly due to inadequate dissection, as very small growing tumors could have been missed. These mice were not classified as "negative." If, however, the graft site was not found in mice sacrificed later than 1 week after inoculation, it was considered likely that the graft had been absorbed. Failure to find a growing graft at that period was unlikely. These mice were classified as "negative."

* This work was supported by a grant from the National Cancer Institute, U.S. Public Health Service.

† Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

Received for publication March 18, 1950.

RESULTS

The data in Table 1 show the incidence of tumor growth in the sacrificed mice. Growth of grafts placed in the anterior chamber was found in 85 of 126 mice (68 per cent). The observation of growing tumor tissue in the anterior chamber of all mice sacrificed at the end of the second week after inoculation also suggests that the "negative" animals sacrified previously had harbored small growing grafts undetected by histological examination.

In contrast, growing tumors were found in only 18 of 93 mice inoculated subcutaneously (19 per cent). The difference between the two groups is significant ($P < 0.001$).

Tumor growth extending beyond the anterior chamber was first observed in mice sacrificed 5 days after inoculation. It was observed in 58 of 61 "positive" mice sacrificed on and after the sixth day. Frequently, it occurred before the anterior chamber was completely filled with tumor tissue. The tumor proliferated readily over both surfaces of the iris and into the vitreous body; it invaded the ciliary body and the pectinate ligament, and extended posteriorly into the choroid and the perichoroidal space. The tumor often extended from the pectinate ligament into the scleral channel used by the anterior ciliary vessels. After penetration of the sclera, growth occurred readily in the periscleral space.

In several animals the tumor appeared partly replaced by granulation tissue. In others, only granulation tissue was found, either limited to the anterior chamber or beyond it, in a distribution similar to that of the growing tumor in the other animals. This was mainly observed in mice sacrificed on the 15th, the 19th, and the 24th days following inoculation and was interpreted as a regression of tumor tissue. Some of these mice still showed foci of mitotic activity of tumor cells and hence were listed in Table 1 as harboring growing tumor. Therefore, the data in Table 1 understate the extent of regression of tumor tissue in mice grafted into the anterior chamber.

Thirty-eight mice with anterior chamber grafts were not sacrificed. In all these the anterior chamber became filled with gray-pink tissue, and the cornea perforated in all but one animal—events which, in the sacrificed animals, were proved to represent tumor growth. The data in Table 2 show that 23 of these animals (61 per cent) developed progressively growing tumors. Metastases were not observed. The other animals developed smaller tumors not exceeding 0.7×0.6 cm. in surface area. They regressed during or after the third week following inoculation.

Forty-three of the subcutaneously inoculated animals were not sacrificed. Six of these (16 per cent) developed progressively growing tumors.

TABLE 1

INCIDENCE OF TUMOR GROWTH IN SACRIFICED MICE

	Grafts into anterior chamber				Subcutaneous grafts		
Time of Sacrifice	No. mice	Growing tumor	Tumor growth limited to ant. chamber	Tumor growth beyond ant. chamber	No. mice	Growing tumor	Graft site not found*
6 hours	10	1	1	0	10	1	8
24 hours	10	1	1	0	9	1	3
2 days	10	3	3	0	9	1	4
3 "	10	7	7	0	9	1	3
4 "	8(9)†	4	4	0	9	0	1
5 "	10	8	6	2	9	1	1
6 "	10	8	0	8	9	0	4
8 "	10	9	1	8	9	3	
10 "	8(9)†	8	0	8	9	2	
12 "	11	11	0	11	9	3	
15 "	10	9	1	8	8	1	
19 "	9	8	1	7	10	2	
24 "	10	8	0	8	9	2	

* See "Materials and Methods."
† Tissue lost in processing.

TABLE 2

INCIDENCE OF TUMOR GROWTH

Transplantation site	Total no. of mice allowed to survive	No. of mice showing progressive tumor growth	Per cent
Anterior chamber	38	23	61
Subcutaneous space	43	6	16

$P < 0.001$.

The difference between this group and that with inoculations into the anterior chamber is significant ($P < 0.001$).

Fourteen of fifteen mice of the ABC strain with anterior chamber grafts developed progressively

growing tumors with large metastases beneath the skin of the neck. This almost invariably occurs in this laboratory, with animals of this strain, following transplantation of C1300 to the anterior chamber. Only two of fourteen mice of the ABC strain grafted subcutaneously with C1300 failed to develop a tumor, while in a third mouse the tumor regressed.

SUMMARY

Mouse neuroblastoma C1300 was transplanted into the anterior chamber of 166 female mice of the C57 brown strain, and subcutaneously into 161. Comparable numbers of mice of each group were sacrificed serially, and the incidence of tumor growth was compared.

Tumor growth was significantly more frequent in mice in which the tumor had been transplanted to the anterior chamber. The tumor grew both within and beyond the anterior chamber.

Progressive tumor growth was significantly more frequent in mice with inoculations into the anterior chamber than in subcutaneously inoculated animals.

Gross metastases were not observed in these mice following tumor transplantation to the anterior chamber, in contrast to mice of the ABC strain in which this event almost invariably occurs following transplantation of this tumor to the anterior chamber.

ACKNOWLEDGMENTS

We are indebted to Dr. Francis D. Gunn and Dr. Jean H. Dougherty of the Department of Pathology, University of Utah Medical School, and to Dr. Murray Shear of the National Cancer Institute for aid in the preparation of this manuscript; to Miss Marie Lettieri and to Mr. Paul Sine for technical assistance.

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Studies on Xanthine Oxidase during Carcinogenesis by *p*-Dimethylaminoazobenzene*

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Protein and riboflavin deficiencies (11, 17, 1, 7) seem to be major dietary factors involved in the production of liver cancers by feeding *p*-dimethylaminoazobenzene. The quantity and type of fat in the diet also appear to be important in this process (14, 18), and other dietary factors, such as biotin, play a role under certain conditions (8, 22). Conversely, a major effect of feeding *p*-dimethylaminoazobenzene is the depletion of liver riboflavin (6, 10, 16). This effect may well be related to its carcinogenic action, since *p*-dimethylaminoazobenzene does not have a similar effect on other tissues, and noncarcinogenic dyes have little, if any, effect on the hepatic riboflavin.

In order for *p*-dimethylaminoazobenzene to be a potent carcinogen, it must act synergistically with a nutritional deficiency. Two important nutritional factors related to this process, protein and riboflavin deficiencies, are the same dietary factors that are capable of depleting liver xanthine oxidase (24, 19, 2). Xanthine oxidase is a complex flavoprotein enzyme (3, 5) and is the only oxidative enzyme involved in the formation of uric acid from nucleotides; it is the only one of the enzymes involved in nucleotide metabolism in rat liver that is appreciably affected by a low protein diet (20). Since these known interrelationships hold many implications of possible importance in the mechanism of *p*-dimethylaminoazobenzene carcinogenesis, a study of the relationship of xanthine oxidase to the production of cancer by *p*-dimethylaminoazobenzene was undertaken.

EXPERIMENTAL

The basic diet used for these studies was identical with the purified 12 per cent casein–5 per cent corn oil diet described by Kline *et al.* (13), except that 1 per cent cod liver oil was added to the diet at the expense of glucose. Two other control diets were the same, but one contained 8 per cent casein and the other 21 per cent casein; an appropriate adjustment of the glucose content was made in each case. These three dye-free diets, varying significantly only in protein content, were fed as controls to each of three groups of 24 Sprague-Dawley male rats weighing 175–200 gm. Three additional groups of 39 rats each were started on the same diets, to which had been added 0.06 per cent *p*-dimethylaminoazobenzene. At the end of 1, 2, and 4 months, six rats from each of the six groups were sacrificed by decapitation for blood and tissue analyses. All the dye-fed rats that survived for 18 weeks (six on 12 per cent casein; fifteen on 21 per cent casein; none on 8 per cent casein) were kept on the same diet without dye for an additional 6 weeks before sacrificing. At necropsy or upon death of the rat a liver section was placed in 10 per cent formalin for histological study; sections were stained with eosin and hemotoxylin. Body weights were recorded every 2 weeks.

Liver xanthine oxidase activity was determined by the method of Axelrod and Elvehjem (2) as previously reported (19). The enzyme activity in spleen, kidney, lung, and intestine was determined similarly (24). The xanthine oxidase activity in rat blood was determined by a procedure to be described, based upon the studies of Kalckar (9); it depended upon a measurement of the disappearance of xanthine and the formation of uric acid during the incubation of a xanthine solution with rat blood. The changes in concentration were determined by measuring the absorption at 270 mμ and 290 mμ.

Determination of xanthine oxidase in rat blood.—The reagents used were: phosphate buffer: 0.0585 M, ph 7.4, Na:K = 1:1; xanthine solution: the same phosphate buffer containing 40–50 γ xanthine per cc.; and 20 per cent perchloric acid.

Fresh phosphate buffer was prepared each time by diluting a concentrated solution. An aliquot of an 0.05 M xanthine solution in 0.05 M NaOH was added to the same buffer before completing the dilution, to obtain the freshly prepared xanthine solution.

1.0 cc. of the xanthine solution was added to a test tube containing 0.2 cc. of defibrinated rat

* This work was done under a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

Received for publication April 3, 1950.

blood. A blank was prepared by mixing 0.2 cc. of blood with 1.0 cc. of the phosphate buffer. Both tubes were incubated for 1 hour at 37°, with continuous shaking. Each was then diluted with 2.8 cc. of water, and the proteins were precipitated by the addition of 2.0 cc. of 20 per cent perchloric acid. After centrifuging thoroughly, to remove the protein precipitate, the supernatant was transferred to a 1-cm. quartz cell and read against water in a Beckman spectrophotometer at 270 mμ, 290 mμ, and 330 mμ. The density reading at 270 mμ indicated the xanthine concentration; the 290 mμ reading gave the uric acid concentration; and the reading at 330 mμ was subtracted from both the 270 mμ and 290 mμ readings, to correct for the turbidity or nonspecific absorption of the solution. The absorption of the blank was subtracted from the corresponding readings of the xanthine-containing solution in order to obtain the amount of xanthine remaining or the uric acid formed. The original xanthine concentration in the reagent used was determined by the same spectrophotometric measurements of a solution containing 1.0 cc. xanthine, 3.0 cc. water, and 2.0 cc. perchloric acid. The difference between these density readings and those calculated to be present after incubation gave the net density changes resulting from the conversion of xanthine to uric acid. The net density changes at 270 mμ and 290 mμ were divided by 0.034 and 0.071, respectively, to convert these figures into μg. of xanthine disappearing per cubic centimeter of filtrate. Multiplying by 6 gave the total μg. of xanthine converted by 0.2 cc. blood; a further multiplication by 5 gave the recorded values in terms of μg. xanthine oxidized by 1 cc. of blood per hour.

The values of 0.034 and 0.071 given above for the change in the density of the solution at 270 mμ and 290 mμ, respectively, that accompanies the oxidation of 1 μg. per cubic centimeter of xanthine to uric acid differ from those reported by Kalckar (9). The differences are due to the shift in the absorption spectra of both xanthine and uric acid in the acid medium of the perchloric acid filtrate. The absorption peak for xanthine in phosphate buffer of pH 7.4 is 277 mμ; in the perchloric acid filtrate it is 264 mμ; the absorption at 270 mμ changes very little with acidification, because the two curves cross at this point. The peak of absorption for uric acid at pH 7.4, 299 mμ, is shifted to 290 mμ in the perchloric acid solution. The values of 0.034 and 0.071 were calculated from the absorption curves for xanthine and uric acid in a $6\frac{2}{3}$ per cent perchloric acid solution.

The phosphate buffer and perchloric acid did not contribute to the absorption at the wave lengths studied. Rat blood filtrates contained considerable material absorbing at 270 mμ and 290 mμ, most of which came from the red cells. Upon incubation of whole blood or serum with phosphate buffer alone for 1 hour there was a slight increase in the absorption at both 270 mμ and 290 mμ. Incubation for 3 hours resulted in a slight decrease in the absorption at 270 mμ and a slightly increased absorption at 290 mμ. These changes indicated a relatively slow conversion of some pre-existing blood constituent to xanthine and then uric acid, but the changes were relatively small after 1 hour of incubation. Xanthine added to blood was recovered quantitatively in the filtrate when the proteins were precipitated immediately. The xanthine concentration in the reagent was unaffected by incubation of the buffer solution alone.

The 330-mμ reading was unaffected by the xanthine or uric acid concentration and was at a point where the absorption by a blood filtrate was minimal and flat. In most cases the correction introduced by the 330-mμ reading was negligible, because both the blank and xanthine-containing solutions from any one blood sample gave nearly identical readings. Filtrates from different blood samples varied appreciably in the amount of absorption at 330 mμ.

Theoretically, the xanthine disappearance could be calculated from the density change at either 270 mμ or 290 mμ, and the two values so obtained should agree. In most cases reasonably good agreement (15 per cent) was observed, and the two values were averaged. In some cases, however, there was a much greater formation of uric acid (Δ at 290) than could be accounted for by the small amount of xanthine disappearing (Δ at 270). In such instances the values were calculated from the density change at 290 mμ only. The reasons for placing greater reliance upon the 290 mμ readings are: (*a*) The change at 270 mμ may be the resultant of some xanthine being formed as well as disappearing during the incubation; (*b*) The blood filtrate blank at 270 mμ is usually much greater than the blank at 290 mμ, and the density change at 290 mμ/μg xanthine oxidized is more than twice that observed at 270 mμ; hence, the change at 290 mμ is relatively greater and more accurate. Repeat determinations on six blood samples that had been stored in the refrigerator for 24 hours gave values that differed from the original determination by 10 per cent or less.

RESULTS

At the time the rats were started on the various diets, nine additional rats from the same group were sacrificed immediately and found to contain 1,188 ± 91 units of xanthine oxidase in the liver.

The changes in the activity of this enzyme in the various tissues that resulted from the diets alone as well as from the inclusion of *p*-dimethylaminoazobenzene in the diets are shown in Table 1. The growth response for each group is also recorded. The effect of each diet is evident at the end of 1 month, and the longer dietary periods do not alter the picture appreciably. Hence, the results will be discussed in approximate over-all average terms; the specific figures, together with the standard errors for the groups, are recorded in the table.

The 21 per cent protein diet maintained the liver xanthine oxidase at about 1,000 units; the dye decreased this value to about 700 units. The 12 per cent protein diet gave levels of approximately 600 units, while only 135 units were found in the corresponding dye-fed rats. The 8 per cent protein diet alone decreased the liver xanthine oxidase to 250 units, and the dye further decreased it to about 100 units. Feeding the dye acted synergistically with a dietary protein deficiency in depressing the xanthine oxidase level in the liver.

The effect of the protein level of the diet on the concentration of xanthine oxidase in the liver is evident and essentially in agreement with previous findings (23, 24). The activity found in the rats fed the 8 per cent protein diet was higher than anticipated, since values approaching zero have usu-

TABLE 1

THE XANTHINE OXIDASE ACTIVITY OF TISSUES OF RATS FED DIETS OF DIFFERENT PROTEIN CONTENT WITH AND WITHOUT *p*-DIMETHYLAMINOAZOBENZENE

Diet	Body wt. (gm.) Start	End	Xanthine oxidase activity (mean ± S.E.)* Liver	Kidney	Lung	Spleen	Intestine	Blood
			(On diet 4 weeks)					
21 per cent Protein	188	332	1312 ±123					60 ±3
"+Dye	182	267	730 ±142					35 ±6
12 per cent Protein	184	267	718 ±153					67 ±5
"+Dye	184	233	175 ±56					32 ±4
8 per cent Protein	183	243	318 ±68					91 ±4
"+Dye	181	191	49 ±21					40 ±7
			(On diet 8–9 weeks)					
21 per cent Protein	193	356	926 ±42	222 ±20	295 ±24	461 ±41	82 ±36	49 ±3
"+Dye	188	324	768 ±117	65 ±36	366 ±46	610 ±98	145 ±69	25 ±2
12 per cent Protein	181	324	413 ±87	156 ±18	261 ±42	368 ±46	15 ±15	53 ±7
"+Dye	191	227	105 ±25	10 ±10	168 ±18	481 ±65	146 ±56	18 ±7
8 per cent Protein	181	233	335 ±48	160 ±55	350 ±38	370 ±57	74 ±26	87 ±9
"+Dye	178	201	114 ±30	7 ±7	225 ±36	435 ±77	81 ±41	19 ±8
			(On diet 16–18 weeks)					
21 per cent Protein	186	449	990 ±34	198 ±29	189 ±28	454 ±22	79 ±31	58 ±1
"+Dye	178	395	616 ±80	7 ±7	165 ±21	431 ±87	29 ±19	42 ±2
12 per cent Protein	182	381	630 ±61	247 ±19	255 ±20	435 ±67	37 ±29	67 ±5
"+Dye	192	312	122 ±15	46 ±19	190 ±24	625 ±40	45 ±45	35 ±4
8 per cent Protein	177	253	82 ±48	200 ±27	244 ±26	421 ±57	54 ±28	91 ±10
"+Dye	202	208	129 ±33	81 ±19	219 ±41	641 ±69	150 ±36	58 ±10

* Tissue xanthine oxidase activity in c. mm. O_2 per gram dry wt. per hour; blood activity in μg. xanthine oxidized per cc. blood per hour. Each figure is the mean for six rats.

ally been obtained with the 8 per cent protein diet used in previous studies (23). The difference is possibly due to the choline deficiency of the diets used for these studies.

The low-protein diets alone did not decrease the xanthine oxidase activity of the kidney, the average level in this series being about 200 units. Feeding the dye decreased this activity to very low levels, an average of 35 units being found for all the diets. The protein content of the diet had no particular influence on the effect of the dye in this tissue. All three diets gave approximately the same results on the lung enzyme—levels of 200–300 units. Inclusion of the dye in the diet gave slightly lower values (approx. 15 per cent). Neither the protein content of the diet nor the dye had much effect on the lung enzyme.

The protein content of the diet had little effect on the xanthine oxidase activity of the spleen, levels of about 400 units being found on all the diets. When the dye was also fed, the enzyme activity of the spleen was generally higher, with an average of 540 units. The dye-fed rats not only maintained normal levels of this enzyme in the spleen, but the spleen was also greatly enlarged over that found on the diets alone. All three diets gave essentially the same low order of activity in the intestine—approximately 60 units. The dye had no additional effect on the intestinal enzyme; occasional increased values were small and of doubtful significance.

The protein content of the diet had an effect on the xanthine oxidase content of rat blood opposite to that of the liver. As the liver enzyme was depleted by the low protein diets, the blood enzyme increased. However, when the dye was also fed, the blood enzyme activity was depressed and held at the same low figure irrespective of the diet. These results are graphically illustrated in Figure 1.

By comparison with the values found for rats maintained on dog chow (24), the purified 21 per cent casein diet decreased the enzyme somewhat in the liver, spleen, lung, and intestine, but not in the kidney. Feeding an 8 per cent casein diet gave a further marked decrease in the liver enzyme but had no additional effect, beyond that of the purified diet alone in the other tissues. The blood enzyme was markedly increased by a low protein diet. *p*-Dimethylaminoazobenzene decreased the enzyme in kidney and blood, irrespective of diet, and decreased the enzyme in liver synergistically with a low protein intake; the dye had no depressant effect on the enzyme in lung, spleen, and intestine. It should be noted that lung and spleen are the two tissues whose xanthine oxidase activities are not increased appreciably by methylene blue (24).

Studies on liver xanthine oxidase.—The following studies were made to assess the method of determining xanthine oxidase in the livers of the dye-fed rats to see if the lower levels of activity were due to an actual absence of the enzyme from the tissue or to the presence of an inhibitor in the liver.

The livers were analyzed in the usual manner and also in the presence of 0.15 cc. of 0.0113 M

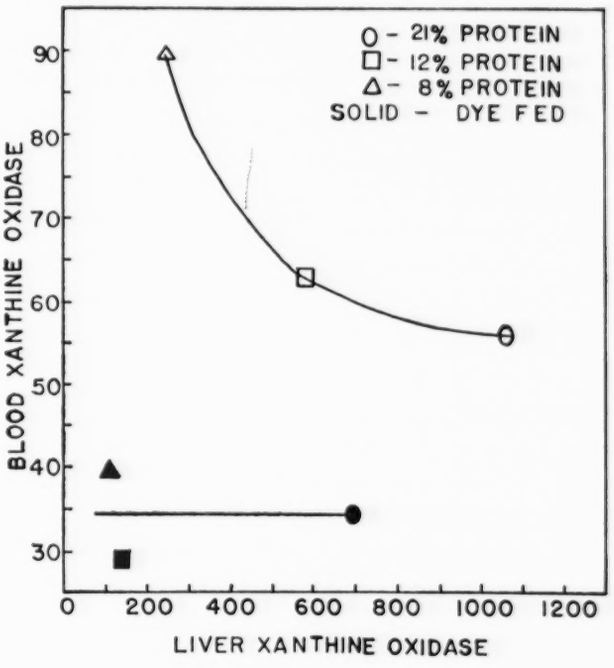

FIG. 1.—The relationship between blood and liver xanthine oxidase in rats maintained on three different diets, with and without *p*-dimethylaminoazobenzene.

Each point represents the average of eighteen rats analyzed after 1, 2, or 4 months on the diets. Liver xanthine oxidase units = c. mm. O_2 per gram dry liver per hour; blood xanthine oxidase units = μg. xanthine oxidized by 1 cc. blood per hour.

methylene blue added to the aerobic system. Purified milk xanthine oxidase (3) was added to the liver homogenate to see if its activity was increased to the same extent in the dye-fed rats as in those on the diets alone. A portion of the liver was homogenized with 2 volumes of water and dialyzed for 3–4 hours against running tap water, and an additional 16–18 hours in a large volume of cold distilled water. The dialyzed homogenate was then diluted with concentrated phosphate buffer and water to give the same proportions used with the original liver. The activity of the dialyzed preparation, as well as the effect of methylene blue and

added xanthine oxidase, was then again determined. Similar studies on normal rat livers were previously reported (19).

The same results were obtained with the rats fed the dye-containing diets for 8–9 weeks and with the rats sacrificed after 16–18 weeks. The latter data, together with the calculated results, have been summarized in Table 2. The following points may be noted: (*a*) Essentially the same enzyme activity was found after dialysis as was present in the original liver in both the dye-fed and dye-free groups. The finding of increased consistent in each group and may indicate the presence of a very small amount of nondialyzable inhibitor of xanthine oxidase in the livers of the dye-fed rats. (*c*) The xanthine oxidase activity of the undialyzed liver homogenate was increased appreciably by the addition of methylene blue to the aerobic system; this increase was consistently greater in the dye-fed rats than in those on the dye-free diets, and the difference was statistically significant in two of the three groups. These results suggest that the auto-oxidizability of this enzyme is somewhat impaired by the dye feeding, since

TABLE 2

THE EFFECT OF METHYLENE BLUE, XANTHINE OXIDASE, AND DIALYSIS ON THE XANTHINE OXIDASE ACTIVITY OF LIVERS FROM RATS FED THE CONTROL AND *p*-DIMETHYLAMINOAZOBENZENE DIETS*†

Diet	Undialyzed				Dialyzed			
	Liver	Liver+ M. blue	Added X.O.	Liver+ X.O.	Liver	Liver+ M. blue	Added X.O.	Liver+ X.O
21 per cent Protein	28	53	18	56	29	35	18	58
"+Dye	18	45	15.5	34	14	27	17	41
12 per cent Protein	18	29	21	47	19	11	18	43
"+Dye	3.5	15	27	37	5	4	20	24
8 per cent Protein	2.3	6.6	24	41	6.5	2.6	23	40
"+Dye	3.0	11	21.5	33	7	3.5	20.5	35.5

CALCULATED DATA

Diet	Liver Activity	Liver+ M.B.	Undialyzed		Dialyzed	
			Liver+M.B. liver	X.O. recovered	Liver+M.B. liver	X.O. recovered
	Dial./undial.	Dial./undial.				
21 per cent Protein	1.04 ±0.04	0.65 ±0.05	1.90 ±0.04	1.52 ±0.04	1.17 ±0.09	1.69 ±0.14
"+Dye	0.76 ±0.15	0.61 ±0.08	2.71 ±0.23	1.08 ±0.14	2.08 ±0.29	1.52 ±0.13
12 per cent Protein	1.10 ±0.09	0.39 ±0.09	1.55 ±0.10	1.41 ±0.09	0.57 ±0.12	1.29 ±0.06
"+Dye	1.51 ±0.35	0.33 ±0.09	4.35 ±0.74	1.31 ±0.13	0.94 ±0.22	0.97 ±0.10
8 per cent Protein	3.52 ±0.90	0.48 ±0.11	3.42 ±0.66	1.59 ±0.09	0.56 ±0.19	1.48 ±0.08
"+Dye	4.10 ±1.60	0.32 ±0.07	5.40 ±2.00	1.41 ±0.14	0.56 ±0.20	1.38 ±0.24

* All original data figures are in c. mm. O_2 per 20 minutes; calculated data are given as ratios.

† Each figure is the average of six rats per group fed the indicated diet for 16–18 weeks. The standard error of the mean is also given for the calculated data.

amounts of activity after dialysis in both 8 per cent protein groups cannot be considered unusual because of the inaccuracy of the method in determining the small amounts of activity in these livers, and because the livers regularly show some activity after dialysis when none was apparent in the original homogenate (19). The responses of the dye-fed and dye-free groups were similar in this respect, so that no evidence was obtained for the presence of a dialyzable inhibitor of xanthine oxidase in the livers of dye-fed rats. (*b*) The activity of the purified milk xanthine oxidase was increased by a factor of 1.50 in the dye-free rats and 1.27 in the dye-fed rats; these figures were unchanged by dialysis. The difference was small but methylene blue acts as an accessory hydrogen transport mechanism between the dehydrogenating portion of the enzyme and oxygen. If the enzyme activities of the livers were considered on the basis of the determinations in the presence of methylene blue, the effect of each diet and the dye would be the same as that already described, but the differences would be smaller. (*d*) The effect of methylene blue on the xanthine oxidase activity of dialyzed liver, which was studied for the first time in this series, was unexpected. For, while there was no loss of enzyme as determined by the usual procedure, methylene blue gave significantly less increase in the activity of the enzyme in the two groups fed 21 per cent protein, and it actually

inhibited the activity of the enzyme in all groups receiving 12 per cent or 8 per cent casein. When the liver homogenates from rats on the 21 per cent casein control diets were dialyzed for 44 hours, some loss of dehydrogenase activity occurred, but the same values were obtained in the presence or absence of methylene blue. This loss of the methylene blue effect after dialysis is not a characteristic of the enzyme, since both the purified milk and liver enzymes show an increased activity with methylene blue after dialysis.

The results clearly show that no dialyzable inhibitor of xanthine oxidase accumulates in the dye-fed rats. There is some indication that a small amount of a nondialyzable inhibitor is present and that it affects primarily that portion of the enzyme which is responsible for its reaction with air rather than the dehydrogenase portion of the enzyme. Dye metabolites are known to be protein-bound in the liver (15). It must also be concluded that under the influence of either a low-protein diet or the dye the liver actually contains less enzyme. The oxidation of urate by all these livers was not inhibited appreciably, especially when the urate was tipped in after 40 minutes (19).

The *in vitro* addition of *p*-dimethylaminoazobenzene to a normal rat liver homogenate had no effect on its xanthine oxidase activity. The metabolism of *p*-dimethylaminoazobenzene by the rat is known to give rise to compounds related to *p*-aminophenol (21); the oxidized or quinimine form of *p*-aminophenol is a potent and relatively specific inhibitor of xanthine oxidase (4).

Endogenous respiration.—The endogenous respiration of the liver homogenate varied with its xanthine oxidase activity as previously described (19) and therefore varied with the protein content of the diet and the presence or absence of the dye in the diet. Figure 2 shows the average oxygen consumption curves for the rats maintained on 12 per cent casein and 12 per cent casein plus dye for 16–18 weeks. The decreased oxygen consumption of the livers from the dye-fed rats is apparent, and it is due to the lowered xanthine oxidase activity in the dye-fed rats. This was determined by a comparison of the average endogenous curve of the livers from rats fed the 21 per cent casein diet plus dye with the curve in Figure 2 for the 12 per cent casein diet alone; both groups of rats had the same average xanthine oxidase activity (17 c mm O_2/20 min), and both had identical endogenous respiration curves. The feeding of the dye had no additional effect on the endogenous respiration curve beyond that which could be accounted for by the decreased xanthine oxidase activity of the livers. Similarly, the rats on 12 per cent casein plus dye, 8 per cent casein, and 8 per cent casein plus dye all had the same low xanthine oxidase activity (approximately 3 c mm O_2/20 min), and all had essentially the same low endogenous curve. The endogenous respiration curves for kidney, spleen, lung, and intestine were not appreciably affected by either the low protein diets or the feeding of the dye. Some small differences of questionable significance were noted.

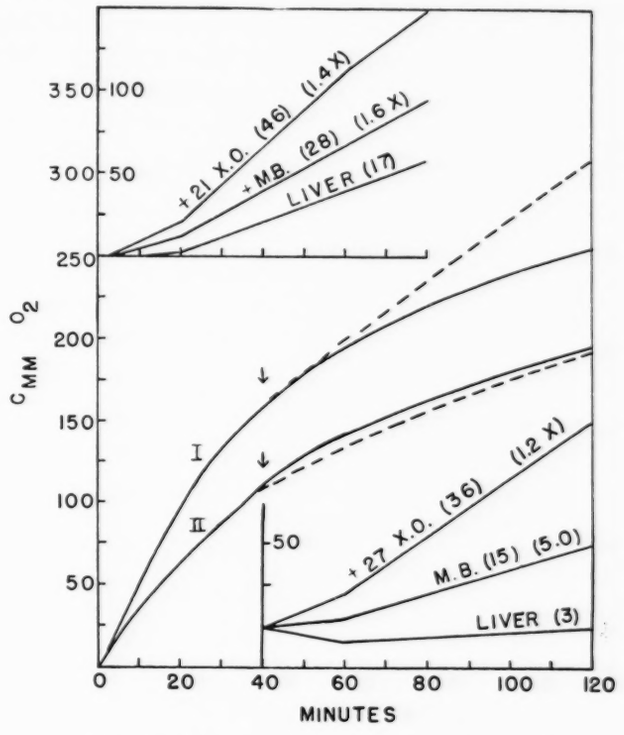

FIG. 2.—The average oxygen uptake curves for liver homogenates from rats fed 12 per cent casein diets for 16–18 weeks.

Each curve is the average for six rats. Curve I = 12 per cent casein diet; Curve II = 12 per cent casein plus 0.06 per cent *p*-dimethylaminoazobenzene. Xanthine was tipped in at the arrows to give the dotted lines. Each Warburg flask contained 1.7 cc. of a 1 + 5 liver homogenate, or 283 mg. fresh liver.

Upper inset shows the net oxygen uptake due to the oxidation of xanthine for Curve I. The effect of added purified milk xanthine oxidase (XO) and methylene blue (MB) to the body of the Warburg flask is also shown.

Numbers along curves refer to c.mm. O_2 per 20 minutes and the ratios by which the activity of the milk enzyme was increased in the liver, or the activity of the liver was increased by methylene blue.

Lower inset shows corresponding values for Curve II.

Pathology.—The liver pathology of rats sacrificed after 1, 2, 4, and 6 months can be summarized as follows. The rats on the 21 per cent casein control diet had essentially normal livers throughout the duration of the experiment. About one-half of the rats on the 12 per cent casein diet had a sig-

nificant amount of fatty infiltration. All the rats on the 8 per cent casein diet had some fatty infiltration with marked fat deposition in over one-half of this group, particularly after 2 months on the diet. Changes characteristic of so-called hydropic degeneration were seen in a few of the animals on the 12 per cent casein diet and in over one-half of those on the 8 per cent casein diet, especially after 2 months. Early progressive cirrhosis was seen after 2 months in only two of the rats on the 8 per cent casein diet. All the above changes are characteristic of what might be expected from the low choline diets of variable protein content.

The most characteristic finding in the group of rats fed the 21 per cent casein diet plus dye was a proliferation of the smaller bile ducts out from the portal area between parenchymal liver cells. This was not accompanied by any inflammatory or fibrotic reaction, and the proliferating cells were non-neoplastic in appearance. This feature was noted with increasing frequency after 1 month on this dye-containing diet. After 4 months of dye-feeding, some animals were continued for 6 weeks more on the dye-free diet alone, and this proliferation was then seen with less frequency, although it still could be seen in a few animals. At the end of 6 months, four of fourteen rats had biliary cystadenomas, and one of these also showed a well developed hepatoma plus a separate bile-duct carcinoma without cirrhosis.

Similar duct proliferation, although with less frequency, was seen in the group fed the 12 per cent casein diet plus dye, and this was accompanied by a moderate amount of fatty infiltration. After 4 months on this dye-containing diet and 6 weeks more on the diet alone, duct proliferation was no longer present, although the fatty change without cirrhosis was present. One animal in this group showed several foci of bile-duct carcinoma without cirrhosis after 6 months.

The addition of dye to the 8 per cent casein diet produced some duct proliferation in addition to the fatty and hydropic changes noted on the diet alone. The duct proliferation was less frequent than was observed with the 12 per cent and 21 per cent casein groups. Occasional progressive cirrhosis was seen after 2 months, and two bile-duct carcinomas were obtained, one at 2 months with slight cirrhosis and one at 4 months with no cirrhosis. No animals in this group survived for a 6-month study.

Changes common to all animals on the various dye-containing diets were seen in the liver parenchymal cells, especially those near the portal area. These cells were characterized by increased nuclear as well as cell size, prominent nucleoli, and a decrease of cytoplasmic granules with increased eosinophilic staining quality of the cytoplasm. These changes were seen with increasing frequency as the time on the diets lengthened. After the dye was discontinued, most of these changes had disappeared at the end of an additional 6 weeks. A few areas of apparently regenerating parenchymal cells were seen, but in no instance could these, with assurance, be considered neoplastic.

DISCUSSION

The synergistic effect of diet plus dye in depleting liver xanthine oxidase activity may be nothing more than a reflection of a more general effect on liver flavoproteins. The depletion of xanthine oxidase and other similar liver flavoprotein enzymes by the diet-dye combination might be the primary effect that allows the carcinogenic process to be initiated. One mechanism by which such enzyme inhibitions could affect the carcinogenic process is through a blocking of the normal degradation of *p*-dimethylaminoazobenzene into noncarcinogenic metabolites (12).

Xanthine oxidase might also be related to the carcinogenic process as one of the enzyme systems involved in nucleoprotein metabolism. With increasing attention being given to the role of the nucleoproteins in cancer, an inhibition of a key enzyme in their metabolism might have more than casual importance in the carcinogenic process. However, the following points do not support this concept: (*a*) this enzyme can be eliminated almost completely from the liver by dietary means alone, without cancer resulting; (*b*) the rats that developed cancer on the 21 per cent casein diet had sub-normal but not depleted levels of liver xanthine oxidase; and (*c*) all these rats excreted normal levels of uric acid and allantoin, showing that nucleoprotein catabolism in the entire animal proceeded adequately in spite of the marked depletion of this enzyme in certain tissues.

Changes in the blood xanthine oxidase suggest another possible mechanism for the action of the dye. As the liver enzyme is depleted by the low protein diet alone, the blood enzyme increases. It seems reasonable to believe that this represents a "leakage" of the liver enzyme into the blood stream. The dye-fed rats show a more severe depletion of their liver enzyme without any increase in the blood enzyme. Does this mean that a protein-deficient diet alters the liver in such a way that it cannot retain an enzyme such as xanthine oxidase, but that the enzyme is being synthesized continuously, nevertheless? Does the lack of elevation

of the blood enzyme in the dye-fed rats signify that the enzyme is not being made? Does this indicate that a fundamental action of the dye is an inhibition of the synthesis of certain liver enzymes, such as xanthine oxidase? Other explanations of these results are equally possible, e.g., the blood of dye-fed rats may contain an inhibitor of xanthine oxidase, or the dye may affect the kidney in such a way that the blood enzyme is excreted.

A comparison of the blood and liver xanthine oxidases, when both are calculated as μg. xanthine oxidized per hour per gram dry weight, shows that a liver activity of 1,000 units is about 24 times as active as the 56 blood units present in the same rats. When the liver enzyme has been depleted to 250 units by a low protein diet, the blood has increased to 90 blood units at the same time, and the liver is then about 4 times as active.

SUMMARY

Rats were fed purified diets containing 21 per cent, 12 per cent, or 8 per cent casein with or without added *p*-dimethylaminoazobenzene. At the end of 1, 2, and 4 months, six rats from each of the six dietary groups were analyzed for xanthine oxidase in the liver, kidney, lung, spleen, intestine, and blood. A method for determining blood xanthine oxidase was described.

By comparison with chow-fed rats, the purified 21 per cent casein diet alone decreased the xanthine oxidase somewhat in the liver, spleen, lung, and intestine, but not in the kidney. The 8 per cent casein diet gave a further marked decrease in the liver enzyme only, and a marked increase in the blood xanthine oxidase.

p-Dimethylaminoazobenzene decreased the xanthine oxidase in kidney and blood, irrespective of diet, and decreased the enzyme in liver synergistically with a low protein intake; the dye had no depressant effect on the xanthine oxidase in lung, spleen, or intestine.

The same xanthine oxidase activity was found in the liver homogenates after dialysis. The activity of added milk xanthine oxidase was increased slightly more in the liver homogenates of dye-free rats than in dye-fed rats. Methylene blue added to the liver homogenate in the aerobic test system increased the xanthine oxidase activity more in the dye-fed rats than in the controls. The effect of methylene blue on the xanthine oxidase activity of the liver homogenate was lost on dialysis, especially with livers from the low protein groups. These results indicated that the livers of dye-fed rats contained a small amount of nondialyzable inhibitor of xanthine oxidase that affected primarily the auto-oxidizable rather than the dehydrogenase portion of the enzyme. The livers also contained less enzyme than the dye-free controls. The effect of the dye in decreasing the endogenous respiration of the liver homogenate was due to a decreased xanthine oxidase activity of the liver.

The low protein diets were characterized by fatty infiltration and hydropic degeneration of the liver. Dye feeding led to bile-duct proliferation and cystadenomas in the 21 per cent casein group and bile-duct carcinomas in the other dietary groups.

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Application of the Seibert Tryptophane-Acid Reaction to the Serum of Malignancy

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In 1947 Seibert, Seibert, Atno, and Campbell (3) reported that the polysaccharide content of serum, as determined by the carbazole reaction of Dische, was increased in tuberculosis, the degree of increase roughly paralleling the degree of severity of the disease. In addition, the average polysaccharide content of the serum was shown to be increased in carcinoma, and in Boeck's sarcoid.

In 1948 Seibert, Pfaff, and Seibert (2), believing the significant polysaccharide to be associated with alpha-globulin, thought that it might be of a nucleic acid nature, since alpha-globulin has been shown to be increased in conditions characterized by tissue destruction. The method of Seymour Cohen (1) for the carbohydrate of desoxyribosenucleic acid consisting of heating with perchloric acid and tryptophane, with production of a pink color, was therefore applied to serum. This reaction is more specific for desoxyribose than is the Dische carbazole reagent. In Cohen's method, desoxyribosenucleic acid is hydrolyzed by the perchloric acid, and the carbohydrate condenses with the tryptophane to give a colored compound. Cohen has shown that, although many substances under these conditions produce colored compounds with tryptophane, of the ones he studied only fructose, in comparable amounts, gives a color similar to that produced by desoxyribose. Whether or not the color produced when the reaction is applied directly to serum is due to desoxyribose has not been determined. The color differs somewhat from that of the desoxyribose compound, and absorption spectra on dialysates from serum fail to reveal an absorption maximum at 260 mμ (2). Fructose is not present in serum in amounts sufficient to account for the reaction (2). Seibert, Pfaff, and Seibert reported values obtained by this method on 134 patients, showing that there was a statistically significant difference between the mean values for groups of minimal, moderately advanced, and far advanced tuberculosis. In fifteen cases of carcinoma the amount of color produced was also above the average value for the normal group. The authors suggested that this test might have usefulness as a diagnostic aid in malignant disease.

Using the procedure of Seibert *et al.*, we have studied 250 cases, as summarized in Figure 1. The results are expressed in Klett colorimeter scale readings as advocated by Seibert. The determinations were carried out as described by Seibert, i e., to 0.25 ml. of serum in 0.75 ml. of 0.9 per cent sodium chloride were added 2 ml. of a 0.25 per cent solution of tryptophane and 3 ml. of 60 per cent perchloric acid. In place of the "serum control" used by Seibert which consisted simply of 0.25 ml. serum in 5.75 ml. of saline, we have substituted a serum control of 0.25 ml. of serum, 2.75 ml. of 0.9 per cent sodium chloride, and 3 ml. perchloric acid. This is then filtered just as are the solutions containing tryptophane in the actual determination. All tubes, both determinations and controls, were read against a blank consisting of saline and perchloric acid.

Several hundred sera have been examined by this method. The results on the 250 given in the following tables and charts are representative. We tried to be as objective as possible in doing the tests. The sera were received from the Robinson Foundation of the University of Pennsylvania, which is conducting a study of the Huggins test.[1] The samples were in numbered tubes, and the person doing the test knew nothing at that time of the status of the patients whose sera were being studied. Huggins tests were also run on the same sera (by the Robinson Foundation Laboratory). A comparison of results by the two tests is to be reported later.

Of the 250 patients reported here, 100 had proved malignancies of various types and locations; 50 had diseases requiring hospitalization, other than cancer or tuberculosis; the remaining 100 constituted our normal group. The normal group requires further explanation: They were

Received for publication April 21, 1950.

[1] To be published.

people who came to the Cancer Detection Clinic for examination and were suffering from many disabilities—most of them minor, but including many cases of hypertension and chronic cystic mastitis. They were, therefore, not healthy, but we felt that they served our purpose better because they represented the particular age group (40–60) on which a test for cancer would be especially valuable.

Because Seibert had reported only 40 normals determined by this method, and because we had made a slight change in the method, we wished to establish our own normal range and used these 100 cases for that purpose. None showed clinical symptoms of malignant disease, and all had a normal Huggins test. None were hospitalized.

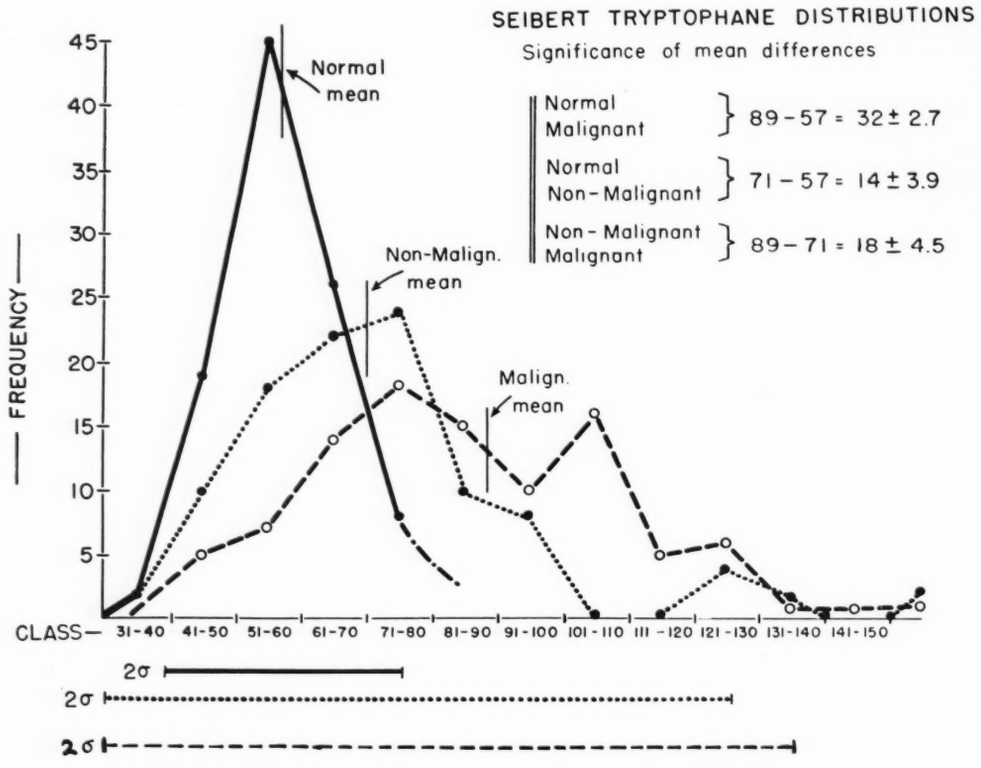

FIG. 1.—Frequency distributions in the tryptophane-acid reactions

RESULTS

In Figure 1 are shown the frequency distributions, range of values, means, and the significance of differences between the means. The differences between the mean values for each of the groups are highly significant. However, the overlapping of values between the groups is so great as to make the use of the test extremely hazardous in the diagnosis of cancer. Our highest normal value was 75, which also represents the upper limit of 2σ. Anything above this value may be considered abnormal, and, if this is so, then 33 per cent of the values of our malignant cases were within the normal range, and a considerably greater percentage of the values for the malignant group were within the range of the nonmalignant disease group. This means that a large percentage (33 per cent) of malignancies will give "false negative" readings.

It is also true that what may be looked upon as "false positive" values (i.e., values above 75) may be found in a certain number of supposedly normal persons. However, with this test we have found, among 200 patients examined in the Cancer Detection Clinic and free of clinical symptoms of cancer, only one with a serum value above 75 (the value for this serum was 76).

We have noted that our percentage of correct diagnoses in cases of malignancy has been decreasing as we have increased the number of patients studied. The same is true in a study of the Huggins test.[1] This may be due at least in part to the fact that we are now examining a larger number of patients on the surgical service who perhaps are in a somewhat earlier stage of their disease than are many of the patients whom we see in the medical wards. Separation of the two groups of patients shows (Table 1) that the percentage of correct diagnoses in the medical group is 74 per cent, in the

surgical group 57 per cent. The correlation of the results of the test with the stage of advance of the disease is being studied further.

Agreement between the results of the Seibert test and of the Huggins test, done on the same sera, is close.[1] The overlapping of the values for the normal, nonmalignant disease, and malignant disease groups are approximately the same for the two tests.

TABLE 1

PATIENTS WITH MALIGNANCY

	Seibert test over 75	Seibert test under 75	Per cent abnormal
Surgical patients (44)	35	19	57
Medical patients (56)	41	15	74

While this work was in progress, Shetlar, Foster, Kelly, Shetlar, Bryan, and Everett (5) reported results of a test utilizing tryptophane and sulfuric acid instead of perchloric. They believed they were determining a mixture of galactose and mannose resulting from hydrolysis of a polysaccharide containing these two sugars plus glucosamine (4). The glucosamine, at the wave length used (500 mμ), was not measured to any great extent. Comparison of the Shetlar and Seibert procedures (4) showed good correlation but not absolute agreement. Use of both Seibert and Huggins tests on the same sera[2] somewhat increases the percentage of correct diagnoses, but even when both tests are used, the diagnosis of malignancy is hazardous.

The use of this test may have a certain limited value in the Cancer Detection Clinic, where abnormal values indicate an abnormality in metabolism which should suggest a re-examination of such patients.

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[2] To be published.

Catalase Studies on Normal and Cancerous Rats*

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Several investigators have studied the effect of tumor growths on the activity of catalase in the liver (7, 8, 13, 16), kidney (7, 8, 9), and erythrocytes (7) of various animals. Brahn (1) originally reported a decreased catalase activity in the livers of humans with malignant growths. Some of the more recent work has been concerned with the effects of inanition (10, 11) and the dietary protein level (16) upon the liver catalase of normal and cancerous rats. Greenstein and associates (9) have reported that a 3-day starvation period leads to no decrease of rat liver catalase, whereas Miller (10, 11) has shown that a 7-day period produces a definite drop in catalase activity which is greater than or approximately equal to the liver protein loss. Weil-Malherbe and Schade (16) have reported that the dietary protein level has little effect on the ability of the tumor to produce drastic reductions of rat liver catalase. Diets containing 8 per cent and 45 per cent casein showed no change in the liver catalase of normal rats, whereas the intraperitoneal injection of sheep serum produced a definite increase in the liver catalase of normal rats. The high level of dietary protein has, however, resulted in increased tumor growth and less severe losses in original body weight of the rat.

The extensive studies of various liver enzymes in cancerous animals by Greenstein and his associates (3–6) and those mentioned above (1, 13, 16), particularly with respect to catalase, have established the following facts with respect to the effect of tumors on the relative activity of catalase in various organs:

1. There is a decrease in liver catalase activity with the progressive growth of the tumor. Within 2–3 weeks after tumor implantation, the activity may be as low as 1/10 to 1/20 of the original value.

2. The effect of the tumor growth is reversible, the liver catalase returning to normal 24–48 hours after removal of the tumor.

3. There may be large individual variations of liver catalase activity in cancerous animals seemingly in the same state of tumor development.

4. The kidney catalase is affected by tumor growth to a much lesser degree than liver catalase. The erythrocyte catalase is not affected.

5. Attempts to find activators, toxic substances, or inhibitors in the blood and other organs have proved fruitless, and it is believed that there has been an actual decrease in concentration of catalase in the liver. This is believed to be due to interference with the synthetic process necessary for maintenance of the catalase concentration in the liver. Recently, Nakahara and Fukuoka (12) have reported a toxic fraction extracted from human tumors which lowers the liver catalase activity in mice when injected intraperitoneally in 100-mg. quantities.

6. The drastic reduction of catalase compared to other liver enzymes and the fact that implantation of growing embryonic tissue and pregnancy (6) do not affect the liver catalase are evidence in favor of the specific ability of the tumor to produce a decrease in liver catalase activity. However, the observations on the effect of the dietary protein level on liver catalase do not preclude the possibility of the nonspecificity of the tumor in producing a decrease liver catalase activity.

The similar structural chemical relationship between catalase and hemoglobin and data in the literature (14, 15), which indicate a possible correlation between the simultaneous occurrence of a decreased liver catalase activity and a severe anemia, suggested that interference with erythropoiesis and catalase synthesis were closely related. This hypothesis might indeed be tested readily by determining the liver catalase activity of animals with various types of anemias. The above facts in conjunction with the effect of inanition (10, 11) and the exaggerated protein requirements of a rapidly growing tumor, as possible causes for decreased liver catalase activity, led to some quantitative studies to determine the changes in the total units of catalase activity in the liver, erythrocytes, and kidneys of normal, cancerous, and protein-depleted rats. An attempt is made to show

* This investigation was aided by a grant from the University of California cancer research funds.

Received for publication April 24, 1950.

variations in the major organs containing catalase when corrections are made for variations in size due to the presence of the tumor or to other experimental conditions.

MATERIALS AND METHODS

Animals.—Adult rats of both sexes of a Long-Evans black-hooded strain were used for all the experimental work. Individual groups were usually selected so that they were all the same age, within very close limits. They were maintained on a routine diet of Rockland pellets with bi-weekly supplements of lettuce or cabbage greens. (The routine diet contained approximately 21 per cent protein.)

The essentially protein-free diet consisted of 80 per cent cornstarch, 15 per cent Wesson oil, 4 per cent U.S.P. salt mixture No. 2, 1 per cent cod liver oil, and appropriate quantities of thiamine, riboflavin, pyridoxine, nicotinamide, niacin, choline, and calcium pantothenate.

Tumors.—All experimental animals were implanted subcutaneously with active fibrosarcoma transplants (Series No. 8), originally induced by subcutaneous injections of methylcholanthrene. Data are available on the amino acid composition of this fibrosarcoma and homologous normal tissue (2). Methods of tumor transplantation were similar to those reported in the preceding reference. One series of experimental animals was implanted with the Jensen rat sarcoma.

Preparation of tissues for analysis.—The animal was anesthetized with ether, and 1 ml. of a 1.5 per cent solution of vital red dye in 0.85 per cent saline solution was injected into the jugular vein using a 27-gauge needle and a tuberculin syringe. The abdominal cavity was exposed, and after approximately 6 minutes the animal was exsanguinated by removing the blood from the abdominal aorta by means of a syringe rinsed with a saline solution of heparin, of suitable concentration to prevent clotting. Aliquots of the blood samples were used as follows: (*a*) A 1-ml. sample for catalase estimation was placed into volumetric flasks and stored in the frozen state. The cells were hemolyzed and appropriately diluted with buffer for the estimation of catalase. (*b*) A 1-ml. sample was diluted to 100 ml. with 0.85 per cent saline, aliquots centrifuged, and the concentration of dye in the supernatant solution compared with a standard using the Klett-Summerson colorimeter with a No. 54 green filter. With reasonable care no significant error is introduced due to hemolysis of erythrocytes. This procedure supplied the data necessary for calculation of the blood volume. (*c*) A ½-ml. sample diluted with 0.85 per cent saline was centrifuged for 30 minutes at 2,500–3,000 r.p.m. to determine the cell volume. Hemoglobin, nitrogen, and hematocrit determinations performed on saline-washed erythrocyte samples of cancerous and normal rats showed that the hemoglobin and nitrogen content could be closely correlated with the volume of packed blood cells. Thus, the hematocrit value or total red blood cell volume would be directly proportional to the corresponding hemoglobin value.

The liver, kidneys, and spleen were removed, rapidly weighed, and immediately placed in a deep freeze unit. The liver and erythrocyte catalase activity was determined on the same day that the animal was sacrificed. Kidney catalase was determined after 1–4 days of storage in the deep freeze, most cases not exceeding 2 days of storage. Preliminary tests indicated that whole liver tissue stored for 1 week showed no loss of catalase activity. As preliminary tests on the catalase activity of spleen and bone marrow of normal and cancerous rats showed no distinctly significant differences, the spleen weight was determined only to find the degree of enlargement associated with tumor growth.

The whole liver was ground 10 minutes at 0° C. with 5 times its weight of M/15 phosphate buffer of pH 7.0 in a small size monel metal Waring blendor. This homogenate was appropriately diluted with the buffer for the catalase determination, and 5-ml. aliquots were taken for the determination of nitrogen by the customary semi-micro Kjeldahl method. The kidneys were homogenized similarly with 25 ml. of phosphate buffer regardless of weight, and similar methods of catalase and nitrogen determinations employed.

Estimation of catalase activity.—The enzymatic activity of the various tissues was determined by measuring the rate of oxygen evolution from H_2O_2 manometrically. The conditions for the determinations were as follows: temperature, 0° C., pH, 7.0; 5 ml. of 1.2 N H_2O_2; 1 ml. enzyme.

All dilutions of the substrate and enzyme preparations were made with M/15 phosphate buffer. The enzyme concentration was adjusted by proper dilution to approximately the same range of activity in order that the ratio of substrate to enzyme concentration was fairly constant. Preliminary investigations were performed with erythrocyte and liver catalase solutions in order to select a substrate to enzyme concentration which showed a constant proportionality with enzyme concentration even with appreciable variations of substrate. The proportionality between enzyme activity and concentration, at any reaction rate over a limited range, makes it possible to express enzyme activity in terms of arbitrary units. The ar-

bitrary unit is defined as that amount of catalase which will liberate 1 ml. of oxygen per second from a 1.0 N H_2O_2 solution at 0° C.

Enzyme and nitrogen determinations were performed in duplicate, the average values of the results obtained being used to calculate the unit enzyme content. The usual rate of oxygen evolution corresponded to approximately 2 ml. per minute, and calculation of the enzyme activity was obtained by determining the maximum initial reaction rate. The error in the catalase determination is less than 5 per cent.

RESULTS

All the pertinent data are given in the tables. Catalase activity is calculated on the bases of nitrogen, fresh tissue weight, total weight of the animal, and a corrected body weight which does not include the weight of the tumor.

Liver catalase activity.—Our experimental results confirm in a general way the observations of other investigators—namely, that rats with growing tumors suffer a loss of liver catalase activity, but the decrease in catalase is neither as large nor as consistent as those reported by Greenstein and coworkers. In no case did liver catalase drop to less than 20 per cent of normal, even in animals where the tumor composed 30 per cent of body weight. And only rarely did we obtain so large a decrease. The failure to obtain as low values as other investigators may be an apparent one, occasioned by a difference in the method used for the determination of catalase activity.

A comparison of Tables 1 and 2 for the UCLA fibrosarcoma and the Jensen rat sarcoma shows very clearly that the catalase loss from the liver is much greater and more consistent in the latter tumor. In fact, when the liver catalase activity is calculated on the basis of units per 100 gm. body weight, as we have done in these tables, a large number of rats bearing the UCLA fibrosarcoma approach the normal value for liver catalase.

It will be pointed out later that usually when the liver catalase is only slightly reduced, other phenomena characteristic of tumor-bearing rats are not apparent.

Erythrocyte catalase.—In the UCLA fibrosarcoma the erythrocyte catalase activity per unit cell volume remains practically the same as in normal animals. The same is essentially true of the rats with the Jensen sarcoma. It should be noted, however, that in the UCLA fibrosarcoma the volume of erythrocytes per 100 gm. of total weight is within the normal range; this is not the case in rats with the Jensen sarcoma. These show a drop in the unit volume of erythrocytes to between 60 per cent and 80 per cent of normal; this, in spite of the relatively large increase in blood volume.

Kidney catalase.—The activity of catalase in the kidneys of cancerous rats suffers a decrease with both tumors. However, when the catalase is calculated in units per 100 gm. of body weight, most of the values fall within the normal range.

Other effects of cancer on the host.—In general, the liver of a tumor-bearing rat is increased in size above that of normal—the increase may be as great as 60 per cent. This is more striking in the Jensen sarcoma than in the UCLA fibrosarcoma. A high degree of spleen enlargement was also observed.

In most cases where the tumor is advancing to the stage where there is a definite decrease in liver catalase, the blood volume of the animal per 100 gm. of total weight is increased 25–50 per cent above the normal values. The erythrocyte volume per 100 gm. total weight either remains virtually normal, as in the case of the rats with the UCLA fibrosarcoma (Table 1), or suffers an appreciable decrease as in the case of animals with the Jensen sarcoma. In either case, the higher blood volume exaggerates the drop in the hematocrit, and it may indicate an apparent anemia when no true anemia exists.

Figure 1 gives a graphic relationship between tumor size as related to body weight and blood volume. It should be pointed out that the cluster of points which do not fit the graph represents rats bearing the UCLA fibrosarcoma; although these animals developed large tumors, their liver catalase was within the normal range. It is regrettable that data were not obtained with the Jensen sarcoma at early and intermediate stages of tumor growth.

Effects of a protein-free diet.—The results of this experiment are summarized in Table 3. All animals placed on the diet suffer a fairly rapid loss of liver catalase. After approximately 10 days of the protein-free diet, the liver catalase stabilizes at approximately one-half the normal value, and it remains there for the duration of the experiment —53 days. Returning the animal from the protein-free diet to a normal diet (21 per cent protein) for 24–48 hours restores the liver catalase to a normal value (Table 3).

It is of interest to note that, although protein-depleted rats show a decrease in liver catalase of the same magnitude as rats with growing tumors, they do not show the other accompanying phenomena of the tumor-bearing rat. The size of the liver, the blood volume, erythrocyte volume, and

TABLE 1

THE EFFECT OF THE UCLA RAT FIBROSARCOMA ON BLOOD VOLUME; ERYTHROCYTE, LIVER, AND KIDNEY CATALASE VALUES

	Sex																Average normal value
	M	M	F	F	F	F	F	M	F	F	F	F	M	M	F	M	
Days since implant	8	8	9	9	14	14	14	20	20	20	22	22	27	27	30	36	
Body wt./tumor wt.	68	86	55	73	13	4.1	5.0	3.3	13	5.4	2.8	5.9	4.9	2.1	2.0	4.2	
Liver wt. per cent total wt.	3.35	3.28	4.20	3.90	4.67	3.59	3.79	4.62	3.80	4.38	3.84	3.62	3.40	3.34	4.0	3.85	3.28
Kidney wt. per cent total wt.	0.71	0.71	0.70	0.83	0.85	0.79	0.83	0.93	0.77	0.74	0.60	0.71	0.68	0.69	0.78	0.71	0.72
Spleen wt. per cent total wt.	0.30	0.33	0.31	0.32	0.79	0.43	0.43	0.60	0.41	0.57	0.39	0.46	0.25	0.29	0.74	0.37	0.23
Blood vol., ml/100 gm total wt.	5.6	5.5	5.5	5.9	6.4	5.8	6.2	7.7	6.3	6.4	7.1	6.2	5.7	7.6	8.4	6.0	5.6
RBC vol., ml/100 gm total wt.	2.2	2.2	2.0	2.3	2.1	1.9	1.9	2.1	2.2	1.8	2.0	1.1	2.2	2.0	2.1	2.1	2.3
RBC catalase activity/mg RBC N	1.07	1.00	1.09	0.96	0.95	0.91			0.99	1.03		0.85	1.10	1.11	0.94	1.00	1.11
RBC catalase activity units/100 gm total wt.	132	123	122	124	112	97			122	104		52	136	124	110	118	143
Liver catalase activity/mg liver N	3.86	4.42	3.60	3.34	1.90	3.88	2.83	3.24	3.68	2.91	2.20	2.12	4.45	2.61	1.44	4.38	4.77
Liver catalase activity units/100 gm total wt.	461	507	500	450	295	477	386	432	491	424	284	255	570	305	187	606	550
Liver catalase activity units/100 gm body wt.	467	515	508	454	319	592	464	563	530	502	386	298	686	448	280	752	550
Kidney catalase activity units/100 gm total wt.	41	27	26	24	22	25	25	35	33	29	23	30	32	26	27	42	36
Kidney catalase activity units/100 gm body wt.	42	27	27	24	24	31	30	45	36	34	32	35	39	38	40	52	36
Liver catalase per cent normal value	85	94	92	83	58	108	84	102	96	91	70	54	125	81	51	137	100

TABLE 2

THE EFFECT OF THE JENSEN RAT SARCOMA ON BLOOD VOLUME; ERYTHROCYTE, LIVER, AND KIDNEY CATALASE VALUES

	Sex										Average normal value
	F	F	F	M	M	F	F	M	M*	M†	
Days since implantation	12	15	15	16	16	16	16	19	21	25	
Body wt./tumor wt.	3.44	3.16	3.74	6.23	5.45	2.72	4.71	4.37	5.36	3.30	
Liver wt. per cent total wt.	3.54	4.14	4.77	3.47	3.76	3.58	5.30	3.28	4.43	4.52	3.28
Kidney wt. per cent total wt.	0.64	0.62	0.71	0.62	0.76	0.65	0.79	0.56	0.79	0.89	0.72
Spleen wt. per cent total wt.	0.45	0.41	0.43	0.55	0.56	0.33	0.45	0.51	0.81	0.78	0.23
Blood vol., ml/100 gm total wt.	7.7	7.6	8.5	5.9	7.0	7.4	8.9	7.1	7.7	7.2	5.6
RBC vol., ml/100 gm total wt.	1.5	1.5	1.8	1.4	1.2	1.7	1.8	1.3	1.8	1.7	2.3
RBC catalase activity/mg RBC nitrogen	1.10	0.95	0.94	0.71		1.04	1.08	0.77	0.96	1.12	1.11
RBC catalase activity units/100 gm total wt.	92	80	95	56		99	109	56	97	107	143
Liver catalase activity/mg liver nitrogen	1.15	2.35	1.69	2.27	2.42	1.54	1.48	0.98	2.16	0.96	4.77
Liver catalase activity units/100 gm total wt.	133	335	245	266	310	184	268	107	314	152	550
Liver catalase activity units/100 gm body wt.	172	440	310	308	366	251	324	131	374	198	550
Kidney catalase activity units/100 gm total wt.		29	27	28	28	34	27	29	39	31	36
Kidney catalase activity units/100 gm body wt.		38	34	33	33	46	33	36	47	41	36
Liver catalase per cent of normal value	31	80	56	56	67	46	59	24	68	36	100

* Last 5 days on approximately 50 per cent protein diet.

† Last 7 days on approximately 50 per cent protein diet.

the size of the kidney remain approximately constant. Kidney catalase activity is definitely above normal, regardless of the method of calculation.

DISCUSSION

Large individual variations in liver catalase occurring in animals apparently at similar levels of tumor growth have been observed by other investigators. At no time, however, have we observed such drastic decreases as those reported by Greenstein and co-workers (7–9) and Weil-Malherbe and Schade (16). There may be a number of reasons for this discrepancy, such as differences in

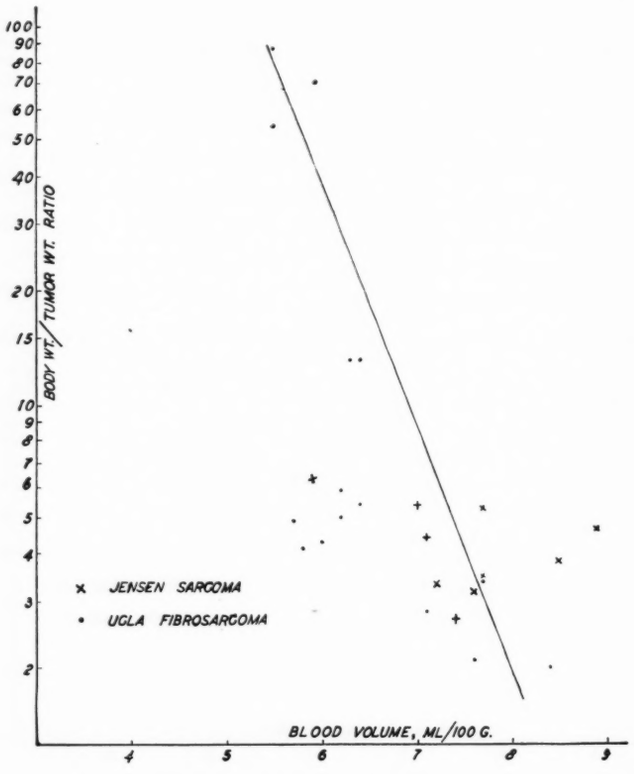

FIG. 1.—The effect of tumor size on blood volume

the strain of rats, the type of tumor used, the diet, and differences in the experimental technic employed. The data reported here were obtained by the use of the Long-Evans strain of hooded rats. It is possible that other strains may suffer a greater loss in liver catalase. Normal rats of other strains, such as August and Wistar, have the same liver catalase activity as the Long-Evans. We have also used two types of tumors. The diet which was used is a standardized commercial diet that is widely used. Therefore, it is our feeling that the observed discrepancy between our results and those of other investigators is due in large part to the different experimental technics used for the catalase determination. We found that liver extracts made with 5 times their weight of phosphate buffer or water suffered losses of activity when stored overnight at 0° C. Recalculating our catalase activity values determined by the method described above on normal rat liver, on the same basis as that given by Greenstein, we obtain a value of 2.9 ml. of oxygen per second for 0.6 mg. total nitrogen at 0° C. compared to 1.7 at 25° C. given by Greenstein.

The differences in the decrease of liver catalase suffered by rats with the UCLA fibrosarcoma, as compared to those with the Jensen sarcoma, are significant. They do not, however, seem to correlate with size of tumor or with the rate of growth as determined by the length of time from implantation to the attainment of a given size. It is conceivable that the two tumors have different "incubation periods," and the actual rate of growth after incubation may be much faster in the Jensen sarcoma than in the UCLA fibrosarcoma. The possibility also exists that the observed differences are due to some inherent characteristic properties of the tumors, independent of growth rate. Whatever may be the reason for the difference in the effect on liver catalase by the two tumors, it is, we believe, significant that a high percentage of rats with the UCLA fibrosarcoma may have a large tumor (between 15 and 30 per cent of body weight), which grew in 3 weeks or less, and still have a liver catalase activity approaching a normal value, when calculated on a body weight basis. While this situation is quite general in the UCLA fibrosarcoma, it is also true in approximately 30 per cent of the rats with Jensen sarcoma. It is felt that this is too general an occurrence to be credited to individual animal variation and therefore should be considered seriously and some other interpretation attempted.

Body weight as a basis for reporting biological phenomena is not an uncommon practice. Nutritional requirements and dosages are almost universally so determined, and more recently enzyme activities have been reported on this basis. We may justify the unconventional use of this basis in this paper, not only because it takes into account the quantitative changes in both the organ being studied and the animal body, but also because it seems to be a more logical basis than merely reporting the activity "concentration" of an enzyme which, although located in a given tissue, seems to have a relationship to the body as a whole. Calculations on a tumor-free weight are presented for comparison and may or may not be justified.

The use of a protein-free diet is one of a number of means attempted to influence liver catalase activity. It has been reported that iron and copper deficiencies in the diet cause decreased liver cata-

TABLE 3

THE EFFECT OF A PROTEIN-FREE DIET ON BLOOD VOLUME; LIVER AND KIDNEY CATALASE VALUES

	Sex															
	M	F	M	F	M	F	M	M	F	F	F	M	M	M*	M†	M
Days on diet	2	2	5	5	7	7	7	9	9	12	12	14	14	17	17	19
Liver, wt. per cent	2.88	3.63	2.92	4.93	2.84	3.14	2.97	3.24	2.99	3.10	3.10	3.31	3.19	4.52	3.78	3.89
Kidney, wt. per cent	0.64	0.61	0.57	0.61	0.61	0.66	0.65	0.75	0.60	0.62	0.56	0.65	0.67	0.83	0.74	0.74
Blood volume, ml/100 gm	5.1		4.9	5.6	5.1	5.2	5.3	5.9	5.6	5.5		5.8	5.4	6.2	6.3	5.8
RBC volume, ml/100 gm	2.6		2.5	2.7	2.5	2.5	2.2	3.0	2.7	2.5		2.1	2.6	2.2	2.5	2.0
Liver catalase activity units/mg N	4.46	2.75	4.83	2.81	3.18	2.94	3.27	3.30	3.33	2.90	2.58	2.38	3.59	1.60	6.42	2.29
Liver catalase activity units/100 gm body weight	408	290	398	351	267	238	298	317	278	238	224	222	260	227	834	235
Kidney catalase activity units/mg N	1.96	1.18	1.74	1.64	1.70	1.74	2.02	1.37	1.88	1.62	2.05	1.58	2.00	1.45	2.44	1.41
Kidney catalase activity units/100 gm body weight	40	22	30	29	32	30	39	30	34	28	35	29	43	35	58	34
Liver catalase per cent normal value	74	53	72	64	49	43	54	58	50	43	41	40	47	42	152	43
Weight loss, per cent of original weight	5	4	4	8	9	5	12	15	11	12	17	16	13	17	14	20

							Sex							Average value protein-free diet	Average normal value
	M	M	M	F	M‡	M	M	F	F	F	M	M	M		
Days on diet	19	21	21	34	36	37	38	41	41	48	49	52	53		
Liver, wt. per cent	2.83	3.21	2.79	2.69	3.82	3.04	2.82	3.23	3.60	2.75	3.43	3.24	2.92	3.25	3.28 (±0.37)
Kidney, wt. per cent	0.68	0.65	0.70	0.68	0.65	0.65	0.63	0.64	0.68	0.61	0.69	0.78	0.72	0.68	0.72 (±0.04)
Blood volume, ml/100 gm	5.2	5.4	5.5		6.3	5.9	5.6	5.3	5.9	4.9	6.0	5.8	5.3	5.7	5.6 (±0.2)
RBC volume, ml/100 gm	2.2	2.2	1.9		2.9	2.0	2.2	1.9	2.3	1.5	2.0	1.7	1.9	2.2	2.3 (±0.1)
Liver catalase activity units/mg N	2.96	2.64	3.55	2.96	4.80	3.00	3.04	3.78	2.95	3.50	3.27	3.86	2.94	3.02	4.77 (±0.56)
Liver catalase activity units/100 gm body weight	220	260	280	250	570	307	250	310	295	266	298	326	245	265	550 (±50)
Kidney catalase activity units/mg N	1.81	2.58	2.04	1.75	2.82	2.11	1.96	2.48	2.24	2.33	2.35	2.46	2.38	1.99	1.73 (±0.23)
Kidney catalase activity units/100 gm body wt.	34	52	38	35	55	39	33	46	41	40	47	51	48	39	36 (±4)
Liver catalase per cent normal value	40	47	51	45	104	56	45	56	54	48	54	59	45	48	100
Wt. loss, per cent of original wt.	19	22	31	42		38	30	31	30	31	41	44	34		

* Final 2 days normal diet.

† Final 4 days normal diet.

‡ Final day normal diet.

lase activity (14). In some preliminary experiments we produced marked liver catalase reductions by producing anemia with a folic acid antagonist, but observed no reduction of erythrocyte catalase. The production of polycythemia by subcutaneous injections of cobalt has also resulted in a decrease of liver catalase activity. Our work on inanition confirms the results reported by Miller (10, 11). Animals starved for 5–7 days suffer a loss of liver catalase of approximately the same magnitude as rats on a protein-free diet. The results are, however, more variable. The most significant result of this experiment is the remarkable constancy of the new level of liver catalase, which develops in 7–10 days and persists as long as the animals are kept on a protein-free diet. It would seem as if this lower limit of liver catalase is a minimum, essential for the survival of the animal. When liver catalase drops below this level, the animal is evidently unable to carry on normal metabolic processes for any significant time. The production of such a situation by growth of a tumor indicates a terminal condition leading to death in a short time.

When the animal is returned to a normal diet, or for 24 hours on a high casein diet, liver catalase returns to a normal value in approximately the same time interval as in the case of a cancerous animal when the tumor is excised. It should also be noted that, although the liver catalase is reduced to approximately 50 per cent of normal, which is fairly close to the average reductions suffered by the tumor-bearing rats, this is not accompanied by the same phenomena that were observed in the case of the tumor-bearing rats. The size of the liver and the blood volume remains essentially normal; the erythrocyte volume suffers a very small decrease, while the kidney catalase appears to increase with increased time on a protein-free diet. These changes are relatively minor as compared to those that occur in the tumor-bearing rat.

The results reported in this paper, as well as many observations on cancerous animals where only one or two factors (blood volume or liver catalase, etc.) were determined and are therefore not included here, prompt us to suggest some possible explanations of the observed phenomena. If we assume that in a normal rat liver catalase is in a steady state, i.e., the rate of its destruction is equal to the rate of synthesis, then it follows that any deviation from the normal is due to a disturbance in this dynamic equilibrium. Thus, in a cancerous rat, or a rat on a protein-free diet, the observed decrease in catalase activity of the liver may be brought about by either an increase in the rate of destruction or a decrease in the rate of synthesis. It is also possible that, in the case of tumor-bearing rats, the rate of catalase synthesis may remain normal or even increase, but the rate of destruction, due to tumor growth, is increased to such an extent that catalase activity continues to drop. In the case of the rats on a protein-free diet both the rates of synthesis and of destruction decrease approximately to the same extent, resulting in a new steady state at a lower catalase activity.

That some catalase synthesis continues in the tumor-bearing rat, even at fairly advanced stages of cancer growth, is evidenced by the fact that in the UCLA fibrosarcoma the erythrocyte volume per unit of total weight is maintained at a fairly constant level with a constant catalase activity. This indicates that new erythrocytes must be formed, since the total weight of the rat continues to increase. It follows, therefore, that both hemoglobin and catalase must be synthesized, because, as it has been pointed out earlier, there does not seem to be a decrease of either hemoglobin or catalase in the erythrocytes of tumor-bearing animals.

One may speculate that there is no relationship between the catalase synthesis in the liver and that in the erythrocytes. These may be quite independent processes. The data obtained with rats on a protein-free diet support this supposition. Here the liver catalase drops to 50 per cent of normal, while the erythrocyte volume and catalase activity remain constant. Since the average life of rat erythrocytes is approximately 30 days, it is evident that those animals in the experiment which were kept longer than 30 days had a complete replacement of their erythrocytes. Thus, the total erythrocyte catalase was either conserved or resynthesized, or the average life period of the erythrocytes was increased.

The suggestion of Greenstein that the tumor "by abstracting from the circulation some material essential to the normal maintenance of the liver catalase" decreases the liver catalase, is not out of harmony with our observations. It is only necessary to add the additional supposition that the substance abstracted is specific for the synthesis of liver catalase and is not essential for the synthesis of catalase in the erythrocytes or the kidney, thus accounting for the numerous instances where only liver catalase is decreased and not the catalase in the erythrocytes or kidney.

SUMMARY

Comparative studies of catalase activity in the liver, kidney, and erythrocytes of normal, cancerous, and protein-depleted rats were made. The results indicate:

1. Liver catalase activity decrease is more severe in the case of Jensen rat sarcoma than in the UCLA fibrosarcoma. In many cases the UCLA fibrosarcoma grew to the same size as the Jensen sarcoma in the same time interval, with the liver catalase activity and erythrocyte volume closely approaching that of normal rats.

2. In our experience with either tumor, liver catalase has not been below 20 per cent of the normal value. This is a less drastic effect than has been described in the literature.

3. Kidney and erythrocyte catalase activity show small decreases, which may very well be within the experimental errors of the methods.

4. Rats with growing tumors which suffer a loss in liver catalase also show an enlargement of the liver and the spleen and an increase in blood plasma. Cancerous rats with normal liver catalase activity show normal blood volumes and normal liver and spleen size.

5. Rats kept on a protein-free diet suffer a drop in liver catalase activity to approximately 50 per cent of normal within 7–10 days from the time they were placed on the diet, and they remain at that level for 53 days or longer. When these animals are returned to a normal diet, the liver catalase activity returns to a normal value within 1–3 days, which is similar to the time required for recovery of tumor-bearing rats after extirpation of the tumor. Kidney catalase activity is slightly higher than normal, while erythrocyte catalase remains normal. Rats on a protein-free diet show no changes in blood volume or liver and spleen size.

6. Evidence for the synthesis of catalase in tumor-bearing rats is shown by the UCLA fibrosarcoma by the constant erythrocyte volume maintained, with increasing total weight of the animal. The suggestion is made that the synthetic mechanism for liver catalase is probably not disturbed. The reduction in the catalase activity may be due to either a more rapid destruction of liver catalase or the abstraction from the blood stream of some necessary constituent by the tumor.

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Some Studies of the Excretion of Diazotizable Material after Feeding 2-Acetylaminofluorene to Rats

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INTRODUCTION

The wide distribution throughout the body of the rat of substantial amounts of material measurable as 2-acetylaminofluorene (AAF), following its ingestion, has been demonstrated by Morris and Westfall (5).[1] Their observations that this distribution was influenced by the intake suggested the probability of considerable variation in the excretion of this carcinogen, perhaps in proportion to the quantity ingested. The development of a sensitive quantitative chemical method for the estimation of AAF in biological materials, by Westfall (6) and Westfall and Morris (7), made possible a study of the excretion of diazotizable compounds in the rat, under conditions of tissue concentrations sufficient to induce cancer when the ingestion was continued for several weeks. The excretion studies reported herein were conducted on intakes of AAF ranging from an amount too low to induce cancer to near the maximum tolerated by the rat.

METHODS AND PROCEDURES

Adult Buffalo strain rats (4) kept in metabolism-type cages were used. The urine was collected in Pyrex glass trays, from which the feces were excluded by a finely meshed galvanized wire cloth attached over the top of the tray.[2]

Loss of fluid by evaporation always occurred under these conditions; hence, direct methods of collection were used to study urinary concentration. Micturition was promoted by holding and annoying the animals. The urine obtained was collected in a graduated glass tube. It was impossible to determine the completeness of emptying of the bladder under these conditions; however, to minimize such effects, no analytical value was retained which represented urine from a mixed sample of less than eight rats, and in addition collections were made on 2 different days.

AAF dissolved in coconut oil was fed to the rats by stomach tube. By this procedure possible contamination of excretatory products with carcinogen from spilled food was avoided. It also insured ingestion of a predetermined quantity of AAF based on the body weight of the rat.

2-Aminofluorene (AF) was determined quantitatively by diazotization of the acid-hydrolyzed acetone extract of the urine according to the method of Westfall (6). The results were calculated as AAF and so expressed.

EXPERIMENTAL RESULTS

A group of Buffalo strain rats was placed in metabolism cages and fed synthetic diet 143 (3). Sixteen mg. of AAF per 100-gm. rat was administered regularly 6 times weekly, by stomach tube, for several weeks before collecting the urine. The urine of ten rats was combined and analyzed for AF, but all values herein are expressed as AAF. The first sample was analyzed 24 hours after the last previous feeding. At this time there was observed a small residual quantity of AAF in the urine, which averaged approximately 5 mg. AAF per 100 milliliter of urine. Following the feeding of AAF there was a rapid rise in the urinary concentration to a peak between 4 and 6 hours after the feeding (Fig. 1). This peak concentration lasted for

[1] The substance(s) found in the tissues or excreted in the urine are measured in terms of AAF, based on diazotization of the freed amino group. Our previously reported data on the solubility of the acetyl derivatives in aqueous solution (6) and Bielschowsky's (1) isolation of the 7-hydroxy derivative from urine are strong evidence that the material fed has been altered in such fashion as to increase its urinary solubility. Our analyses depend on an intact, diazotizable amino group in the residual molecule and do not show whether the latter has been altered, e.g., to the hydroxyl, sulfate, or glycuronate, etc., derivative(s). The diazotizable derivatives, however, are calculated in terms of AAF as the standard of reference. The reader should keep the above limitations in mind.

Received for publication April 29, 1950.

[2] No preservative was used for the urine collected for the weekly intervals owing to the fact that all the commonly used ones (e.g., thymol, toluene, merthiolate, etc.) interfered with the subsequent estimation of the diazotizable material. Furthermore, Bielschowsky and Green (2) have shown that some fluorene derivatives are bacteriostatic. The urines collected directly were analyzed immediately. AAF added to normal urine plus a small amount of food material (to simulate spillage of dietary material) and exposed for a week in the laboratory could be recovered quantitatively.

approximately 2 hours, following which there was a gradual decline in concentration of AAF in the urine, and 24 hours after administration the concentration of AAF had almost reached the initial level.

The rapid increase in urinary concentration of AAF followed by a slower decline over a long period suggests that the rat may have been able to render considerable amounts of the carcinogen no longer detectable by diazotization. This possibility was subjected to experimental test by setting up several groups of ten animals each and feeding them different levels of AAF, while simultaneously varying the frequencies of administration. These animals were all fed by stomach tube either 3 or 6 times weekly. The animals were fed AAF for several weeks prior to the 1-week collection periods. The quantity of AAF fed during each weekly period varied from 0.25 to 16.0 mg/100 gm rat. The results of the studies on excretion in this experiment are illustrated in Figure 2. At a level of intake of 0.25 mg. AAF per week, designated as D3, only a trace of AAF was recovered in the urine—which suggests that the animal is largely converting that amount of carcinogen to a form no longer diazotizable. Increasing the quantity of

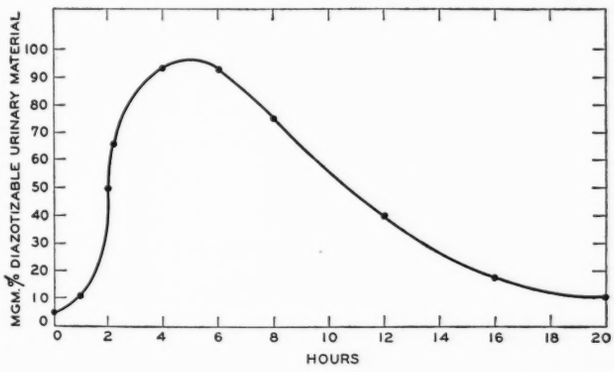

FIG. 1.—Concentration of diazotizable AAF in rat urine during a 24-hour period.

carcinogen fed increased step-wise the quantity of excreted AAF, as illustrated by columns C3, B3, and A3. The amount recovered as AAF was 10, 29, and 45 per cent, respectively.

The per cent of the dose recovered in the urine was also affected by the frequency of administration. This is illustrated in the two columns labeled A6 and A3 (Fig. 2). The total amount of AAF fed both groups was identical. This amounted to 16 mg/100 gm rat/week. When this quantity of carcinogen was ingested in six equal doses weekly, approximately 28 per cent was recovered in the urine. When the same quantity of AAF was ingested in three equal doses weekly the quantity recovered in the urine approximated 45 per cent (Fig. 2). It was concluded from this experiment that the ingestion of very small quantities of AAF results in a very slight elimination of the diazotizable compound in the urine; when the total intake of AAF was very large the urinary excretion was greatly increased but did not exceed 45 per cent of the intake; less AAF was excreted in the urine when a large dose was

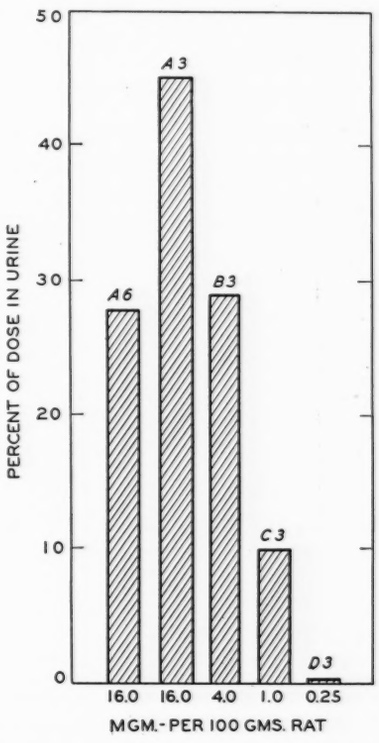

FIG. 2.—The per cent of the weekly dose of AAF recovered in the urine after feeding variable amounts either 3 or 6 times weekly by stomach tube, as indicated by the number at the top of each column.

given daily than when the same quantity was given thrice weekly.

The excretion of AAF into the urine for a period of several weeks was next determined. A group of ten rats was placed in metabolism-type cages and fed by stomach tube 16 mg AAF/100 gm body weight/week. This amount was administered in three equal doses weekly. The urine was collected and analyzed for each week for a period of 5 weeks. Twenty-two per cent of the ingested dose was excreted during the first week, 25 per cent for the second and third weeks, 33 per cent for the fourth week, and 28 per cent for the fifth week (Fig. 3). Slightly less AAF was excreted during the first week than during the subsequent 4 weeks, but the difference is not significant. There is no indication, therefore, that the rat is less able to excrete diazotizable derivatives after several

weeks of ingesting the compound than during the first week of ingestion.

The excretion of diazotizable compounds in the urine of the rat following the final dose was measured. The rats were confined in metabolism-type cages and the urine collected and analyzed for AAF after each 24-hour period. The rats had ingested AAF for several weeks prior to the collection period. They were fed by stomach tube 16 mg AAF/100 gm rat/week. The total dose of AAF ingested by this group of rats was 90 mg. Thirteen

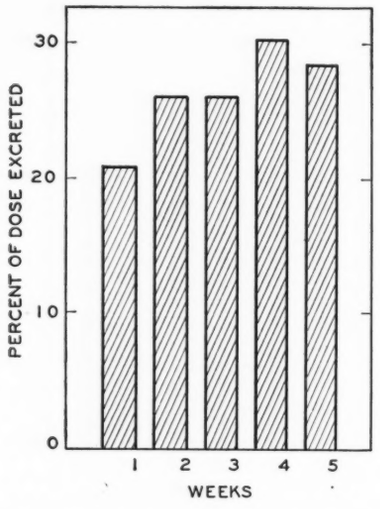

FIG. 3.—The average per cent of a 16-mg/100-gm body weight dose of AAF excreted in the urine weekly.

mg. was found in the urine on the first day (Fig. 4). This rapidly decreased daily for a period of 6 days. On the sixth day only 2 mg. was present in the 24-hour period. Small amounts were excreted during the next 3 days, with none detected on the ninth day. The data do not indicate how much of the ingested AAF was present in, or excreted into, the digestive tract of the animals during the period of collection. It is presumed that AAF remained in the digestive tract for some time after the beginning of the urinary collection. The results indicate rapidly decreasing elimination of diazotizable compounds in the urine of the rat for a period of less than 9 days following a single dose.

DISCUSSION

The data of Morris and Westfall (5) on the distribution of AAF in the rat after a single dose show a peak concentration in the plasma, liver, and kidney between the third and fifth hours after administration, followed by rapid decline during the next 10 hours. The data presented in Figure 1 are also in good agreement with the concentrations observed in the body. If a rat is fed a single feeding of AAF there occurs a high concentration of the diazotizable material in the body during the first few hours, followed by a gradual depletion of the tissues during the next several hours.

One analytical value of 156 mg. per cent was obtained from collecting the urine of rats at the 6-hour period. Ninety per cent of this collection came from two of the eight rats used. It was not included in the data presented in Figure 1, because it was not representative of the group. It seems worth noting, however, that this high value shows that the apparent plateau of the curve of Figure 1, occurring between 4 and 6 hours, is not to be attributed to a limiting solubility of the urinary compound. Bielschowsky, after feeding AAF to rats (1), has isolated 2-acetylamino-7-hydroxyfluorene from the urine. Bielschowsky's metabolite

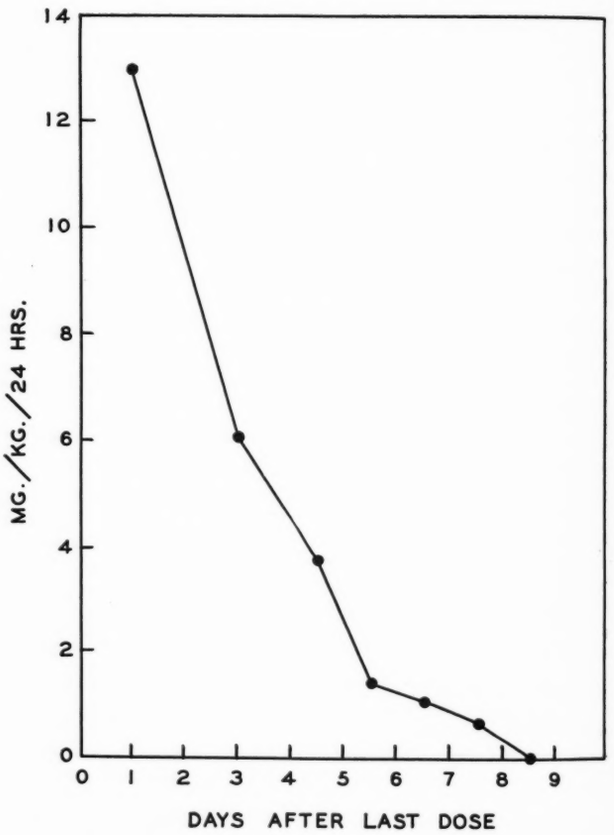

FIG. 4.—The rate of urinary excretion of AAF after a final dose.

has a solubility similar to this urinary compound, but after previous hydrolysis and diazotization it gives a weak color-reaction when R-salt is used as the coupling reagent (1).

Significantly larger amounts of diazotizable AAF were recovered in the urine when the same total amount was ingested in three equally divided weekly feedings than when it was administered in six equal feedings. This probably can be ascribed

to a higher concentration of the carcinogen occurring in the body of the rat during the first few hours after feeding the larger quantity. More diazotizable material apparently spills over into the urine under such conditions, because the animal is unable either to excrete it through the bile or otherwise to convert the larger quantities to a nondiazotizable compound.

This view receives added support from the experiments on urinary excretion when the amount ingested was varied from 16 to 0.25 mg/100 gm rat/week, but the number of feedings was identical. The amount of AAF recovered from the urine was proportional to the dose, even when the amount ingested (0.25 mg.) was noncarcinogenic.

Approximately the same quantity of diazotizable compounds was recovered each week for 5 weekly periods when the intake was kept constant. This may indicate a constant rate of conversion of the ingested material to other pathways of excretion.

Approximately 30 per cent of a 90-mg. dose was excreted in the urine of rats as diazotizable material during a period of 8.5 days following the last dose. It is believed that this portion represents the carcinogen not converted to other as yet unknown complexes. Some could have been eliminated through the bile and intestinal tract. If such is the case, these complexes are no longer detectable by the diazotization method. The results do not distinguish between AF and AAF, and both could have been present in the urine.

SUMMARY

2-Acetylaminofluorene (AAF), in solution in coconut oil, was fed by stomach tube to rats, under different conditions. The acid-hydrolyzable, diazotizable material excreted in the urine was estimated colorimetrically as 2-aminofluorene but calculated as AAF.

There was a rapid rise in urinary concentration following a final dose which reached a peak between 4 and 6 hours. There was a slower decline in concentration during the next 14 hours until the base level was reached at approximately 20 hours. Only a trace of diazotizable material was recovered from the urine following the ingestion of 0.25 mg/week/100 gm rat, divided into three equal feedings. Increasing the dose to 1.0, to 4.0, and to 16.0 mg/week resulted in a direct increase in urinary excretion which reached a maximum of 45 per cent on the highest dose. When the 16.0-mg. dose was administered in six instead of three weekly feedings, the amount found in the urine amounted to only 28 per cent.

Rats uniformly excrete in their urine approximately a third of the AAF ingested as diazotizable compound weekly for several weeks, and approximately one-third of a final feeding was recovered in the urine during the subsequent 8.5 days. None was detected thereafter.

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Inhibition of Nucleic Acid Synthesis by Folic Acid Antagonists*

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Woolley and Pringle (13) have recently reported the formation of 4-amino-5-carboxamidoimidazole during growth of *Escherichia coli* in the presence of 4-aminopteroylglutamic acid (aminopterin). Some of us have observed that excessive doses of folic acid speed up the leukemic process in mice and that folic acid will reverse the well known anti-leukemic action of aminopterin (11). This reversal of the anti-leukemic action of folic acid antagonists by means of large doses of folic acid had previously been reported by Burchenal (4) and Law (7).

In the face of the above information, the suggestion that formyl folic acid might be involved in introduction of a single carbon unit into purines (5) and the observation that formate is a precursor of the 2-carbon atom of uric acid in pigeons (12), certain postulates were made regarding the rate-controlling function of folic acid in leukemia (11). This unoriginal hypothesis, based largely on Gordon's observations (5), suggested that folic acid might be involved in the last step of biosynthesis of the purine skeleton, that step being the transfer of formate via formyl folic acid to the 2-position of the purine skeleton. Thus, if the requirement for purines (for formation of cellular nucleoprotein) was greater in the more dynamic leukemic cell-forming tissue than in normal cells, then an excess of folic acid might be expected to speed up the leukemic process, and a deficiency of this vitamin (or co-enzyme) caused by a folic acid antagonist might preferentially depress malignant mitosis.

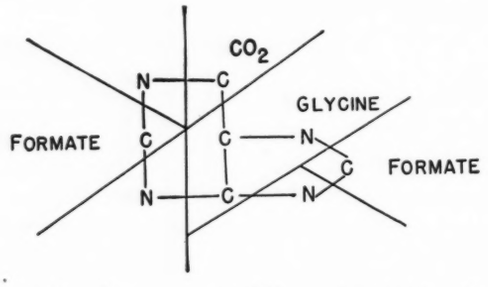


FIG. 1.—Possible precursors of the purine skeleton (2, 10)

The present series of experiments was designed to study the effect of well known folic acid antagonists, aminopterin and A-methopterin (4-amino-N^{10}-methylpteroylglutamic acid), on the incorporation of formate and CO_2 into nucleic acids and nucleic acid purines of normal mice.

EXPERIMENTAL

To ascertain the rate at which formate carbon and CO_2 are incorporated into the nucleic acids and nucleic acid purines (Fig. 1) of normal mice, control experiments have been carried out with carbon 14-labeled sodium formate and sodium bicarbonate. Groups of four mice each were injected with the labeled compounds and immediately placed in a metabolism chamber. After a period of 6 hours, the animals were sacrificed, and the viscera (liver, spleen, testes, kidneys, washed intestines, and pancreas) pooled and mixed with an equal weight of viscera homogenate from non-injected mice. An aliquot of the mixed viscera homogenate (from Waring blendor) was saved for direct oxidation and activity assay. From the remainder, the combined nucleic acids and/or the desoxyribose nucleic acid (DNA) were isolated using the procedure of Plentl and Schoenheimer (8) and Brues, Tracy and Cohen (2), respectively. Aliquots of the nucleic acids obtained were assayed for C^{14} content. The remainder of these samples was used for isolation of the nucleic acid purines as the silver salts. These twice-precipitated silver purines were also measured for radioactivity. All such measurements were carried out by a gas phase procedure which has already been described (10).

* This work was supported by the American Cancer Society, upon recommendation of the Committee on Growth of the National Research Council; the Research Fund of the Southern Research Institute; and Mr. Ben May, Mobile, Alabama. Certain data reported herein were obtained in a program supported by the Atomic Energy Commission, Division of Biology and Medicine. The authors wish to acknowledge the technical assistance of Mrs. Constance Nolan, Miss Linda Simpson, Miss Margaret Ann Newton, Miss Martelia Bell, and Mrs. Janita Chapman.

Received for publication May 6, 1950.

In additional experiments, mice (groups of 4 each) have been injected daily with the maximum tolerated dose of aminopterin or A-methopterin for 6 days; on the seventh day these mice were given an LD_{50} dose of the anti-folic acid compound and were then injected with $HC^{14}OONa$ (1.4 μc.) or $NaHC^{14}O_3$ (11.8 μc.), immediately placed in a metabolism chamber, and at 6 hours the pooled viscera subjected to the isolation procedures mentioned above. The objective of these experiments has been, of course, to observe the effect of folic acid antagonists on the incorporation of formate and CO_2 into the nucleic acid purines. The results obtained are set down in Tables 1 and 2. All values have been multiplied by 2 to correct for addition of inactive viscera homogenate.

DISCUSSION

It seems apparent from the data presented in Tables 1 and 2 that the multiple injection of normal mice with a folic acid antagonist (aminopterin or A-methopterin) profoundly decreases the rate of synthesis of nucleic acids. The fact that administration of aminopterin did not inhibit CO_2 fixation into the control tissue suggests that the nucleic acid effect may be a somewhat specific inhibition, at least within the period involved. Carbon dioxide is now considered a member of the pool of glycogen precursors and is known to be rapidly incorporated into amino acids and fats. Thus, over-all tissue CO_2 fixation serves as a sensitive rate index for a number of metabolic processes. In Experiment No. 13, where a single injection of aminopterin (an LD_{50} dose) was administered to mice immediately before injection of $NaHC^{14}O_3$, no significant decrease in nucleic acid or nucleic acid purine synthesis was observed. This might be considered preliminary evidence that multiple injections of the folic acid antagonists are necessary to produce a folic acid deficiency which, in turn, is responsible for the failure in purine synthesis. The folic acid antagonists in question reduced the specific activity of the viscera of formate-injected mice by only about 25 per cent, while incorporation of formate carbon into nucleic acid purines was reduced almost 15-fold. The decrease in incorporation of CO_2 into nucleic acid purines following treatment with folic acid antagonists is interpreted to indicate that CO_2 is incorporated into a purine precursor which, because of the purine inhibition accompanying treatment with these compounds, is never completed and thus never enters into purine anabolism.

TABLE 1

THE EFFECT OF AMINOPTERIN ON INCORPORATION OF FORMATE INTO NUCLEIC ACIDS AND NUCLEIC ACID PURINES (1.4 μC. INJECTED INTO ALL MICE)

Experiment no.	Treatment	Specific activities (μc/mol carbon) Viscera homogenate	Combined nucleic acids	Combined nucleic acid purines
1	Control	8.48	57.6	138.4
2	Control	4.42	71.8	176.8
3	Control	5.14		
4	Control	5.16		
Average		5.8	64.7	157.6
5	Aminopterin*	4.32	6.20	10.82
6	A-methopterin†	4.4	4.7	9.17

* Mice injected at the level of 0.23 mg/kg on 1, 2, 3, 4, 5, and 7 days and at the level of 8.33 mg/kg on the eighth day (immediately before injection of active formate).

† Mice injected at the level of 3 mg/kg on 1, 2, 3, 4, 5, and 7 days and at the level of 90 mg/kg on the eighth day (immediately before injection of active formate).

TABLE 2

THE EFFECT OF AMINOPTERIN ON INCORPORATION OF C^{14} FROM $NaHC^{14}O_3$ INTO NUCLEIC ACIDS AND NUCLEIC ACID PURINES (11.8 μC. INJECTED INTO ALL ANIMALS)

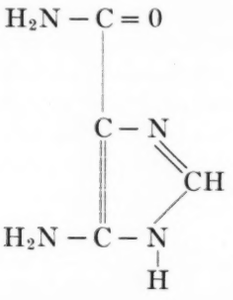
Experiment no.	Treatment	Specific activities (μc/mol carbon) Viscera homogenate	Combined nucleic acids	DNA	Combined nucleic acid purines	DNA purines
7	Control	0.68				1.41 (0.48)
8	Control	0.77				1.76 (0.44)
9	Control	0.90	1.65 (0.54)	1.95 (0.46)		2.10 (0.44)
10	Control	0.71	2.32 (0.31)	2.22 (0.32)		
11	Control	0.98	2.50 (0.35)		3.27 (0.3)	
12	Control	0.99		2.63 (0.38)		
Average		0.87				
13	Aminopterin*	0.88	1.71 (0.52)	1.236 (0.71)		2.20 (0.40)
14	Aminopterin†	1.32	1.05 (1.26)		0.54 (2.45)	

* A single injection of 8.33 mg/kg immediately before administration of $NaHC^{14}O_3$.

† Injection at the level of 0.23 mg/kg on 1, 2, 3, 4, 5, and 7 days and at the level of 8.33 on the eighth day (immediately before administration of $NaHC^{14}O_3$).

NOTE: The values in parentheses are the ratios of the specific activities of original viscera homogenate to the various fractions isolated therefrom.

The results obtained by Woolley and Pringle showing the build-up of the following compound by *E. coli* when inhibited by aminopterin:



4-amino-5-carboxamidoimidazole

along with the present observation that aminopterin or A-methopterin-treated mice are greatly inhibited with respect to nucleic acid purine synthesis (formate utilization) strongly suggests that folic acid, possibly acting as formyl folic acid or folinic acid is involved in completing the purine skeleton.

The seemingly most logical assumptions which might be made at the present time are that folic acid or folinic acid attached to an active center (protein, see Allfrey and King [1]) is acting as an enzyme or co-enzyme in formate transfer. Perhaps aminopterin and A-methopterin compete for this center and, when given in excess, prevent the formation of enough of the folic acid complex to allow for purine synthesis. Another possibility is that a direct inhibition of the synthesis of pteroylglutamic acid might be a result of administration of folic acid antagonists.

It has been shown that folic acid given in excess will speed up the leukemic process and a deficiency of this metabolite will preferentially inhibit leukemic cell division (11); therefore, since neoplasms apparently synthesize nucleic acid more rapidly than most host tissue, it would appear that one might explain the mechanism of action of folic acid antagonists in cancer chemotherapy on a purely differential rate basis as suggested by Hitchings *et al.* (6).

It also seems possible that the mechanism discussed above may be involved in the well known hematopoietic response observed in certain types of anemia following folic acid therapy.

SUMMARY

Aminopterin and A-methopterin, well known folic acid antagonists, have been shown to inhibit the incorporation of C^{14} from formate into nucleic acids and nucleic acid purines. Under the conditions of these experiments, incorporation of $C^{14}O_2$ into purines was partially blocked, but no decrease was observed in tissue CO_2 fixation. The relationship of these observations to the mechanism of action of folic acid in purine synthesis and the anti-leukemic mechanism of "folic acid inhibitors" is discussed.

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Relative Susceptibility of Various Organs to Tumor Transplantation*

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Metastasis of neoplasms is a complex process involving several steps. First, is the separation of cells from the main tumor; then follow invasion of a vessel, transport, arrest, penetration of the vessel wall, and, finally, establishment and growth in the new site with new stroma and vascular supply. Some aspects of this process have been discussed in recent papers from this laboratory: (*a*) the conditions governing the release of tumor emboli from the primary tumor (13) and (*b*) the distribution of tumors in various organs after tumor emboli have been artificially introduced into the blood stream (2).

The present investigation is concerned with a third aspect of metastasis, the comparative ability of various organs to support tumor growth—that is, to supply tumor cells with stroma, adequate blood supply, and a generally suitable environment. This problem may appropriately be studied by implanting fragments of tumor in various organs and observing incidence of "takes" and rate of growth. If the tumors fail to grow in a particular organ, or grow better in some organs than others, the ability of a given organ to support tumor growth might be regarded as an important factor in determining the distribution of metastases. If, however, it should be found that implanted tumor fragments grow equally well in all organs, then the distribution of metastases would likely depend on other factors.

There are reports in the literature of the growth of tumor implants in the spleen (1), liver (8), muscle (3), kidney (7), adrenal (4), and several other organs, but none allows a comparison of the growth in different organs of the same animal.

To obtain a generally valid answer to the question of relative organ susceptibility, several tumors and several species of animals were used: A mouse sarcoma, a rat carcinoma, and two rabbit carcinomas were implanted in appropriate hosts.

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, Public Health Service.

Received for publication May 6, 1950.

MATERIALS AND METHODS

Tumor fragments of approximately equal size, 2 mm. in diameter, were inserted through a knife-slit into the organs indicated in the accompanying table. Fibrosarcoma 241 was implanted into C57 mice; Walker rat carcinoma 256 into Long-Evans hooded rats; V_2 squamous-cell carcinoma into male mixed breed rabbits; and Brown-Pearce carcinoma into male mixed breed rabbits.

To ascertain if the presence of a pre-existing tumor alters organ susceptibility, a fragment of Walker 256 tumor was inoculated subcutaneously into the flank of Long-Evans hooded rats. One week later, the tumors were implanted, as in the previous series. After suitable intervals the animals in all series were sacrificed, and the individual tumors inoculated into the organs were weighed. The weights of the tumors in the several organs were then compared.

TABLE 1

FIBROSARCOMA 241 IN 21 C57 MICE*

Organ	No. of "takes"	Average tumor weight (gm.)
Muscle	19	0.637
Kidney†	7	0.637
Liver	21	0.593
Spleen	21	0.575

* Time of sacrifice: 8th–14th day, 11th day average.
There is no significant difference in size between the tumors in the above organs. For example, when muscle and splenic tumors are compared, the difference and its standard error are 0.062 ± 0.498, and $P = 0.9$. This means that an equal or lesser difference would occur by chance 90 times out of 100 and is not statistically significant.

† Only seven mice had tumors implanted into kidney.

RESULTS

The mouse sarcoma was implanted into muscle, kidney, liver, and spleen, and, as shown in Table 1, grew equally well in all these organs, the average weights of the tumor being close to 0.6 gm.

A similar result was obtained with Walker carcinoma 256 in hooded rats, implanted into muscle, kidney, liver, and spleen; there was no significant difference in tumor growth in these organs (Table

2), the average weights of the tumors being approximately 1 gm.

However, when this carcinoma was implanted into rats already bearing a subcutaneous tumor, the growths in muscle were larger than in liver and kidney (Table 3). It is unlikely that this difference is of much significance, as it can probably be explained by a fortuitous blood supply of the tumors in muscle; many of the implanted tumors were located near the surface of the muscle from which they invaded the subcutaneous tissue and so secured an additional set of blood vessels.

TABLE 2

WALKER 256 CARCINOMA IN 17 HOODED RATS*

Organ	No. of "takes"	Average tumor weight (gm.)
Muscle	17	1.18
Kidney	17	1.14
Liver	17	1.09
Spleen	17	0.85

* Time of sacrifice: 12th day.
There is no significant difference in size between the tumors in the above organs. For example, when muscle and splenic tumors are compared, the difference and its standard error are 0.33 ± 0.19, and P = 0.10.

TABLE 3

WALKER 256 CARCINOMA IN 27 HOODED RATS BEARING SUBCUTANEOUS TUMORS*

Organ	No. of "takes"	Average tumor weight (gm.)
Muscle	25	1.69
Spleen	27	1.21
Kidney	25	1.05
Liver	27	0.95

* Time of sacrifice: 4th–15th day, 12th day average.
The tumors in muscle are significantly larger than those in liver (difference and its standard error 0.74 ± 0.31 and P = 0.03) and kidney (difference and its standard error 0.64 ± 0.30, and P = 0.04). A possible explanation of these differences is found in the text. There are no other significant differences.

TABLE 4

V_2 CARCINOMA IN 27 RABBITS*

Organ	No. of "takes"	Average tumor weight (gm.)
Spleen	27	12.03
Muscle	24	4.64
Adrenal	27	4.23
Kidney	23	4.00
Liver	22	1.51

* Time of sacrifice: 9th–36th day, 21st day average.
Splenic tumors are significantly larger than all others, e.g., compared with muscle, the difference and its standard error are 7.39 ± 2.70, and P < 0.01. Also, liver tumors are smaller than those in muscle (difference and its standard error 3.13 ± 1.29, and P = 0.03). The values presented above include necrotic material. The results are unchanged when only viable tumor is considered.

Different and surprising results were obtained with the rabbit tumors. The V_2 carcinoma grew much better in the spleen than in muscle, adrenal, kidney, and liver (Table 4), averaging 12 gm. in weight, as compared with the next greatest weight, 4.6 gm.

It was this predominance of growth of V_2 carcinoma in the spleen that led to the use of a second kind of rabbit tumor, the Brown-Pearce carcinoma, to find whether the massive growth of the splenic tumors depended on some peculiarity of V_2 carcinoma or whether another tumor would also grow better in the spleen than in other organs. By implanting Brown-Pearce carcinoma in the same set of organs, it was found that growths in the spleen were definitely larger than those in kidney and muscle (Table 5).

TABLE 5

BROWN-PEARCE CARCINOMA IN 26 RABBITS*

Organ	No. of "takes"	Average tumor weight (gm.)
Spleen	25	10.07
Adrenal	26	5.27
Liver	24	3.78
Kidney	25	3.11
Muscle	25	3.09

* Time of sacrifice: 10th–21st day, 15th day average.
Splenic tumors are significantly larger than those in kidney (difference and its standard error 6.96 ± 3.44, and P = 0.037) and in muscle. Muscle tumors are significantly smaller than those in adrenal (difference and its standard error 2.18 ± 0.84, and P = 0.035) and spleen. There are no other significant differences. The results are not altered when only viable tumor is considered.

Thus, in mice and rats no difference was found in the ability of the various organs tested to support growth of implanted tumor fragments. In rabbits, however, a distinct difference came to light—growth being greatest in the spleen with the V_2 carcinoma; with the Brown-Pearce carcinoma a similar, though less definite, result was obtained.

DISCUSSION

These experiments show that implants of four kinds of animal tumors are able to evoke a new blood supply and stroma and grow almost equally well in each of the organs tested. The only exception noted was the rabbit spleen, which seemed to support tumor growth better than most other organs. This is surprising, since spleen (and muscle) have been thought to represent poor "soil" (9) for the growth of metastases—secondary tumors in these organs supposedly occurring rarely. However, Warren and Davis (11), reviewing the incidence of secondary tumors of the spleen in man, found that in widespread dissemination of metastases the spleen was involved about as frequently as any other organ. They inferred that the spleen has no anti-neoplastic qualities, as had often been suggested, and that the apparent rarity of secondary growths in that organ probably is due to the failure of sufficient numbers of tumor emboli to reach it. Saphir and Parker (10), Herbut and Gabriel (5), and Harman and Dacorso (6) present similar evidence. The experimental evidence in

animal tumors has been reviewed by Woglom (12) and also supports this view.

The results of the present experiments and the fact that almost all organs, including spleen and muscle, offer little resistance to the direct extension of cancer, suggest that scarcity of metastases in certain organs is unlikely to be due to inability of such organs to support tumor growth or to provide it with stroma and an adequate blood supply. Rather, the explanation for scarcity of metastases in these organs should be looked for either in (*a*) failure of tumor emboli to reach the organs, (*b*) failure of tumor cells to be arrested, or (*c*) inability of tumor cells to escape from vessels.

SUMMARY

Tumor fragments were transplanted into several organs (kidney, liver, adrenal, spleen, and muscle) of three different species to ascertain whether some organs support tumor growth better than others. Fibrosarcoma 241 was implanted into C57 mice, Walker rat carcinoma 256 into Long-Evans hooded rats, V_2 carcinoma and the Brown-Pearce tumor into rabbits. Animals were sacrificed at appropriate times and their tumors removed and weighed. The tumors grew in all organs tested. In the mice and rats no differences were found among the weights of tumors in the various organs. In rabbits the tumors of the spleen were generally larger than those of other organs.

One step in the process of metastasis of tumors is acquisition by the tumor of a vascular stroma and subsequent growth in the new site. This aspect of metastasis may appropriately be studied by the method of transplantation used in the present experiments. Application of these results to the problem of metastatic tumor growth suggests that scarcity of metastases in certain organs depends on factors other than inability of such organs to support the growth of the tumor.

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Titration of the Milk Agent Virus in Milk and Lactating Mammary Gland Cells*

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The milk agent virus involved in mammary carcinogenesis in mice has been demonstrated to be present in high concentrations in both the lactating mammae and mammary carcinomas of infected mice. The injection of as little as 10^{-5} to 10^{-6} gm. equivalents of lactating breast tissue has been sufficient to infect suitable test animals,[1] as judged by their subsequent development of mammary cancer (1, 4). Although data from previous experiments would suggest that this virus multiplies rather rapidly during the growth of mammary cancer through successive transplants (3), the present study was undertaken in an attempt to obtain data concerning the rate of duplication of the virus during the period of lactation. Observations in this laboratory indicate that during mid-lactation the mammae of mice produce about twice their own weight in milk each day. Such an outpouring of fluid containing a high concentration of virus would soon lead to a great reduction of virus in the gland tissue, unless viral duplication was proceeding at a rather rapid rate. If it were found, therefore, that the concentration of the virus remained high in the gland during lactation and yet was essentially as high in the milk as in the glandular tissue, it would seem to be evidence indicative of a rapid multiplication of the virus during lactation.

In the course of such an experiment, data as to the relative merits of milk and lactating breast tissue as source materials for the purification of the virus would also be obtained.

* Assisted by grants from the Minnesota Division of the American Cancer Society, the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, and the Graduate School of the University of Minnesota.

[1] Since three major factors are necessary for the development of mammary cancer in mice, such animals must be genetically susceptible to the development of breast cancer and have a proper hormonal constitution but lack the milk agent.

Received for publication May 26, 1950.

MATERIALS AND METHODS

Since the original titration experiments reported by Barnum *et al.* (1), several other experiments have been carried out in this laboratory, the results of which clearly show that the concentration of milk agent in the glands of different lactating females, even when they are from the same inbred stock, may vary widely when tested under uniform conditions. The end-point of such titration series (the greatest dilution at which approximately 50 per cent of the test animals develop breast cancer) has varied from 10^{-4} to 10^{-6} gm. equivalents. It is evident, therefore, that for this type of an experiment the milk and the glandular tissue must be obtained from the same donor animals.

The mammae of two strain A female mice after 15 days of nursing their third litters were used for source material. The young were removed from the pens the evening before the females were sacrificed. It has been observed repeatedly that the overnight accumulation of milk more than doubles the weight of the mammae, indicating that during mid-lactation these glands produce approximately twice their own weight of milk each 24 hours.

The donor mice were killed by ether anesthesia and their mammae dissected free of the skin and subcutaneous musculature. The glands were collected in a beaker in an ice-water bath, forced through a chilled tissue press having 1-mm. openings, and collected on a chilled watch glass. Throughout the entire fractionation procedure which followed great care was taken to maintain the solutions at a temperature approaching 4° C. The milk was drained from the gland pulp and, after dilution with 3 volumes of alkalinized saline,[2] was centrifuged at 2,000 × *g* for 30 minutes to insure removal of any glandular cells that might have been included during decantation. A pellet of

[2] 1 cc. of 0.1 N NaOH per 500 cc. 0.85 per cent NaCl.

submicroscopic particles was then obtained by a 90-minute centrifugation at 23,000 × *g*.

The gland pulp was washed twice with alkalinized saline to remove the major portion of the contaminating milk, and was then ground in a cold mortar, without the addition of abrasive, to break the cellular membranes of the gland cells, while leaving the nuclei intact. Four volumes of alkalinized saline were then added, and the connective tissue remnants, uncrushed gland cells, and the freed nuclei were removed from the suspension by a 4-minute centrifugation at 1,500 × *g*. This extract of the mammary gland cell cytoplasm was then spun 30 minutes at 2,000 × *g* to remove the large granule fraction containing the mitochondria after which the microsome pellet was obtained by a 90-minute centrifugation at 23,000 × *g*. As a washing procedure, the microsome pellets obtained from both the gland cell cytoplasm and from the milk were resuspended in alkalinized saline and resedimented at 23,000 × *g* for 90 minutes.

The materials injected into test animals were the washed microsome fraction from the gland cell cytoplasm and the whole milk, after the 2,000 × *g* clearing run. Dilutions were prepared in M/60 phosphate buffer at pH 7.0–7.1, and each test animal received 0.5 cc. of solution injected intraperitoneally. The time elapsing from the sacrifice of the donor animals to the injection of the test animals was 7–8 hours.

The test animals employed were young ZBC female mice lacking the milk agent produced in the following manner:

Ax ♀ (A strain lacking agent) × Zb ♂ (Z or C3H lacking agent)
↓
AxZbF1 ♀ × Zb ♂
↓
ZBC

All animals were 23–26 days of age at the time of injection, and litter mates were so distributed that no group of mice receiving the same injection contained more than one animal from any given mother. The 29 AxZbF1 females used to produce these test animals were observed until their death (average age at death was 21.1 months), and none developed breast cancer, attesting to their freedom from the milk agent.

After injection, the test animals were bred to Zb males for approximately 6 months. All the young were killed shortly after birth, so that the average number of litters per test animal was 4.4. All animals developing subcutaneous masses were autopsied, and only those on which a positive histological diagnosis of mammary cancer was made were recorded as having developed mammary cancer.

RESULTS

The development of mammary tumors in the test animals is recorded in Table 1. It would appear from these data that the milk agent virus was present in approximately the same concentration in the milk as in the secreting gland cells. Actually, a somewhat greater per cent of the animals receiving the injection of 10^{-6} gm. equivalents[3] of cleared milk developed breast cancer, but the number of mice in each group was, of necessity, small. Furthermore, it should be remembered that the microsome pellet from the gland tissue had been sedimented and manually resuspended twice prior to the preparation of the dilute solutions for injection, making a completely even dispersion of infective particles very unlikely. Since the end-point appears to be the same for the two starting materials, combining the data to increase the number of animals in each dilution group seems justified. When this is done, much of the variation disappears, and the end-point of the titration becomes rather sharper. Whereas a constant per cent of the animals receiving the three lower dilutions developed cancer, there was a sharp decline in the incidence of tumors in the group injected with 10^{-7} gm. equivalents of material.

TABLE 1

DEVELOPMENT OF TUMORS IN TEST ANIMALS RECEIVING INTRAPERITONEAL INJECTION OF LACTATING MAMMARY GLAND PREPARATIONS

Fraction	Gram equiv. injected	No. mice/no. developing tumors	Per cent Ca	Av. tumor age (days)
Tissue Microsomes	10^{-5}	15/13	86.7	388
	10^{-6}	14/6	42.8	403
	10^{-7}	14/4	28.6	421
Milk	10^{-4}	15/11	73.4	349
	10^{-5}	15/10	66.7	395
	10^{-6}	12/11	91.7	404
	10^{-7}	10/2	20.0	579
Combined data	10^{-4}	15/11	73.4	349
	10^{-5}	30/23	76.6	391
	10^{-6}	26/17	65.4	404
	10^{-7}	24/6	25.0	469

Chemical studies of several of the fractions were carried out, and some of these analyses are given in Table 2. The isolation and chemical fractionation of the mammary gland microsomes in a number of experiments has yielded reproducible re-

[3] Throughout this paper the dilutions are expressed in terms of the original starting material. Thus 1 gram equivalent of gland microsomes is the amount of microsomes prepared from 1 gm. of milk-free breast tissue.

sults, and the figures obtained in this experiment are representative. Considerable variation has been encountered in working with mouse milk, particularly in the chemical fractionation, according to the Schneider procedure (7). In this particular experiment the milk "microsomes" contained only 0.386 mg. of trichloroacetic acid-precipitable nitrogen per gram of cleared milk. Other experiments indicate, however, that milk particles with the general sedimenting characteristics of "microsomes" and "large granules" possess considerably more acid-soluble than acid-precipitable nitrogen. The amount of this soluble nitrogen left in solution seems to depend to some extent, however, upon the relative amount of acid-precipitable nitrogen present in the starting material.

TABLE 2

CHEMICAL ANALYSES OF FRACTIONS PREPARED FROM LACTATING MAMMARY GLANDS

Fraction	Total N (mg/gm*)	Lipid P (mg/gm)	PNA-P (mg/gm)
Tissue microsomes	2.32	0.231	0.247
Cleared milk	11.79	0.086	0.0675

* The designation mg/gm refers to the milligram of a given constituent found in the fraction isolated from 1 gm. wet weight of tissue.

An intensive study was undertaken employing the electron microscope in an attempt to visualize a characteristic particulate body in microsomal preparations from mammary tissue containing the agent which was not present in similar preparations from uninfected mammae. During the course of this study the publication of Porter and Thompson (6) appeared, describing a characteristic particle in epithelial cells of mouse mammary carcinomas grown in tissue culture. Particular attention was therefore directed toward a search for particles that might correspond to those illustrated by these investigators.

Throughout these studies the resuspended microsomes were diluted in M/60 phosphate buffer at pH 6 8–7.4, formvar-coated screens were floated on small drops of the preparations, and these drops were then exposed to osmium tetroxide vapor for approximately 30 minutes. The preparations were freed from salt by floating them on distilled water, after which they were blotted and dried over phosphorus pentoxide. The preparations were examined either directly, depending upon the staining properties of the osmium to produce sufficient contrast, or they were shadowed lightly with heavy metals, as described originally by Williams and Wyckoff (8).[4]

The microsomes from gland tissue and from milk differed greatly in morphology, as can be seen in Figures 1 and 2. Although many preparations from this and other experiments were studied, no morphologically distinct particle could be identified in preparations from infected mice that was absent from uninfected animals. Occasionally, particles of approximately the size and general shape and density of those described by Porter and Thompson were encountered in these preparations, but they seemed to occur as frequently in glands lacking the agent as in those possessing it (Fig. 2). Also, the bizonal appearance described by Porter and Thompson was not noted in these rather infrequently occurring particles.

The failure to identify a characteristic particle in the microsomal fraction from the mammae of infected mice should in no wise be construed as evidence indicating that the particles encountered in the cultured mammary carcinoma cells do not represent the milk agent virus. Rather, it would seem to indicate that the centrifugally prepared fractions of the mammary glands that we have examined contain relatively so few virus particles that they are overlooked among the mass of normal tissue microsomes. Using the electron microscope as a tool to help us follow visually the purification of the milk agent virus from such very impure preparations does not seem, therefore, to be feasible.

DISCUSSION

Since in mid-lactation the mammae of mice produce at least twice their own weight of milk each day, the finding of the milk agent virus in concentrations in the milk equal to that in the mammary gland cells suggests that the virus is multiplying rather rapidly during this period. A more extensive and conclusive investigation of this particular

[4] The authors wish to acknowledge the technical assistance of Dr. J. F. Marvin during this portion of the investigation.

FIG. 1.—An electron micrograph of the "microsome" fraction obtained from the milk used for injection in the titration experiment reported here. The discrete spheroid particles range in size from 250 mμ down to 50 mμ or less.*

FIG. 2.—An electron micrograph of the microsomes from a lactating mouse mammary gland that lacks the milk agent. In such microsomal preparations, be they from infected or noninfected glands, the majority of the particles appear flattened, very probably as a result of the method of preparation. The few spheroid particles seen here are in the size range of the "characteristic particle" described by Porter and Thompson but lack the bizonal character described by them.

* These electron micrographs were taken by Dr. Keith Porter of the Rockefeller Institute for Medical Research, to whom the authors wish to express their sincere thanks. The shadow-casting was done with chromium at an angle of 12°.

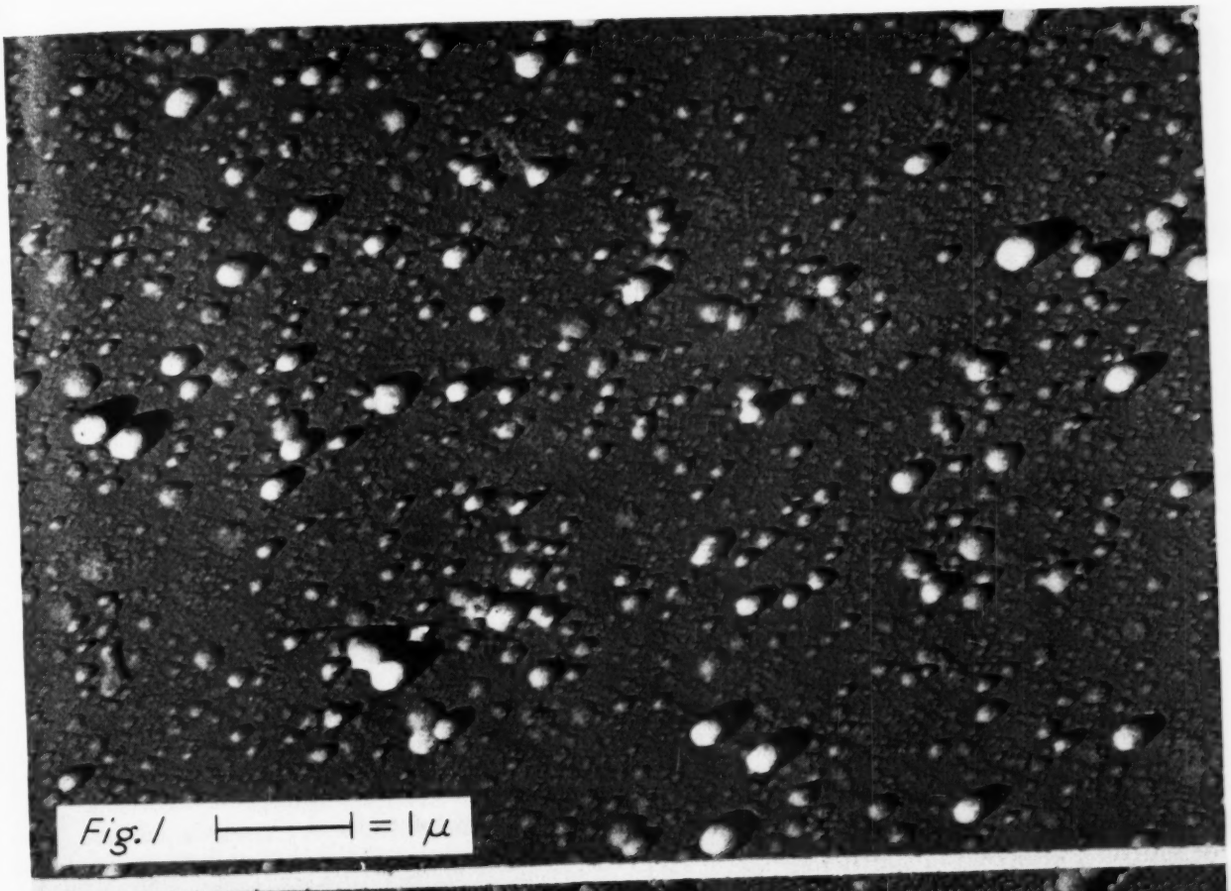

Fig. 1 ⊢——⊣ = 1 μ

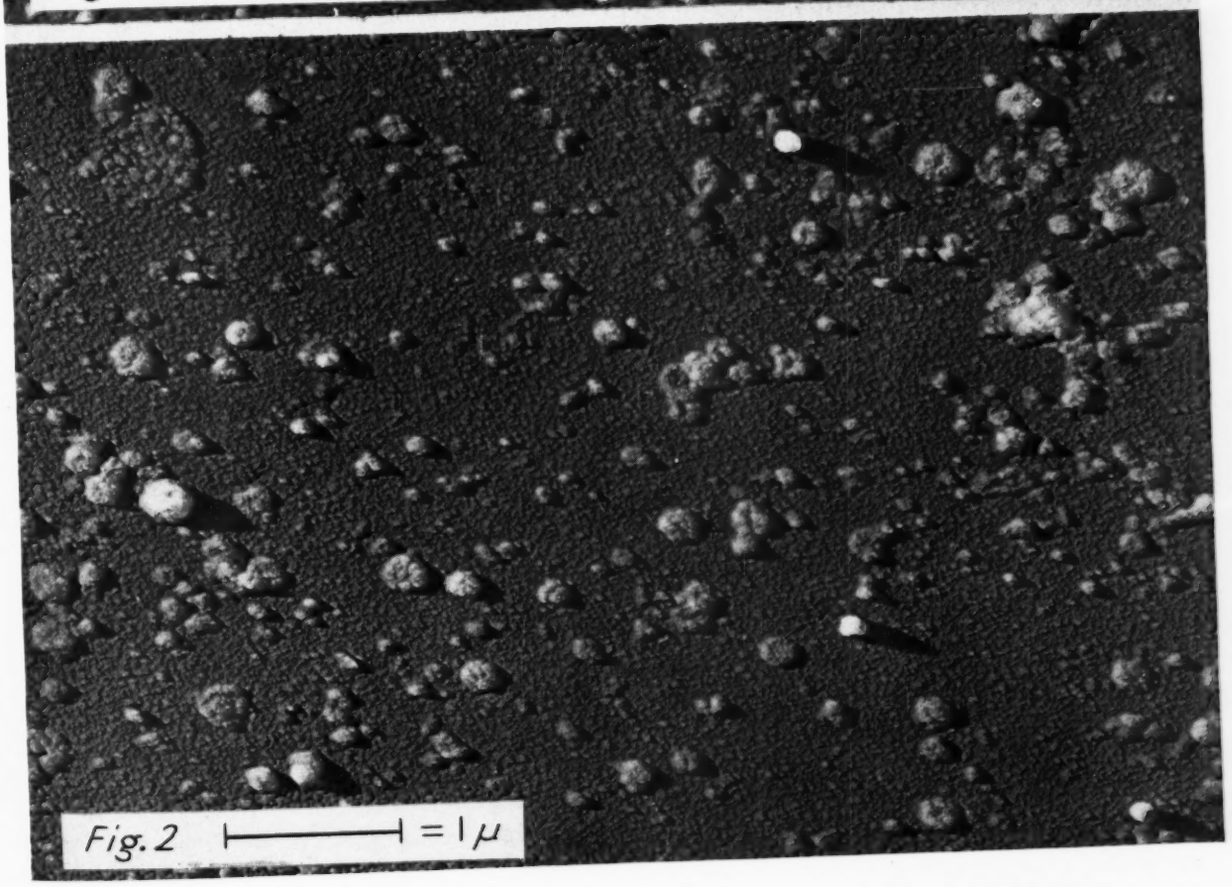

Fig. 2 ⊢——⊣ = 1 μ

phase of the problem is complicated by the great variation in viral content of lactating mammae of different mice of the same inbred strain, as indicated by several titration experiments carried out in this laboratory under as nearly comparable conditions as possible. However, the data recorded here would seem to encourage the use of those technics of viral investigation, such as the cultivation of the virus in embryonated eggs, that depend upon a rapid duplication of the virus.

These results also indicate that, as far as viral content is concerned, milk is as good a source material as is the gland tissue itself. It is, therefore, of some interest to compare the relative infectivity of the impure preparations employed in this study with that reported by Graff *et al.* (5) for a purified material prepared from milk. These authors have as yet reported no serial titration studies, but they do indicate that 46 per cent of a group of test animals developed mammary cancer when injected with a suspension of purified material containing 10 γ of nitrogen. They further state, "We have obtained carcinomas after the injection of as little as 8 mγ of nitrogen." In this study the animals injected with 10^{-6} gm. equivalents of mammary gland microsomes received but 2.32 mγ of nitrogen, and yet 43 per cent of the animals developed tumors and some tumors appeared in the animals receiving one-tenth this amount. The minute amounts of pentose nucleic acid contained in each injection at the end-point of the titration is also of some interest. Whereas 10^{-6} gm. equivalents of tissue microsomes contain 0.247 mγ of PNA phosphorus, previous experiments have shown that more than 95 per cent of this microsomal PNA can be eluted without destroying infectivity (2).

It should be emphasized that microsomal preparations such as this are probably composed for the most part of normal cell constituents with only a small component of virus, as indicated by the similarity of chemical composition of microsomes prepared from infected and noninfected mammae.[5] Our inability to find a characteristic particle in the microsomal fraction prepared from lactating mammae containing the milk agent also suggests a great impurity of these preparations as far as viral content is concerned.

[5] C. P. Barnum and R. A. Huseby, unpublished data.

SUMMARY

In this titration experiment, the concentration of the milk agent virus was found to be as great in the milk as in the mammary gland cells producing that milk. This has been interpreted as indicating a rather rapid duplication of the virus during lactation.

Some chemical analyses are given which indicate that at the dilution considered to be the 50 per cent end-point of the titration each animal received 2.32 mγ of microsomal nitrogen and 0.247 mγ of PNA phosphorus. The probability that the microsomal fraction is very impure, as far as the milk agent virus is concerned, is emphasized.

An investigation employing the electron microscope failed to reveal a characteristic particle in these impure preparations.

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Book Reviews

Biological Actions of Sex Hormones. By HAROLD BURROWS. London and New York: Cambridge University Press, 1949. Pp. 615. $8.50.

This second edition of *Biological Actions of Sex Hormones* has been entirely revised and reset. This has made possible the inclusion and co-ordination of pertinent information reported in the literature since 1945, when the first edition was published. The organization of the subject matter for purposes of presentation is essentially the same as that of the first edition. This organization is logical and quite satisfactory.

This book of 23 chapters is divided into the following six main parts: (1) Gonadotrophins of the pituitary and placenta. This part is concerned with the nature and functions of gonadotrophins and gonad-pituitary interrelationships. (2) Gonadal hormones. This is essentially a general review in which biological tests, chemical structure, sources, functions, excretion, factors influencing the action of gonadal hormones, antagonisms, tissue growth in reference to cancer, and other effects such as their anesthetic properties are dissussed. (3) Androgens. Gradients and variation of androgenic action, androgen action on the reproductive organs before and after their complete differentiation, and their action on accessory reproductive organs and other tissues are reviewed. (4) Estrogens. The action of estrogen on embryonic and differentiated gonads, the anterior pituitary gland, accessory genital organs, the mammary gland, and the influence of estrogen on the development of mammary cancer and on general growth processes are reviewed in considerable detail. (5) Progestins. This part is concerned with the development and maintenance of corpora lutea, the action of progestin on the pituitary, vagina, uterus, and mammary gland, and with the co-operation and antagonism between progestin and estrogen. (6) Sex hormones of the adrenal cortex. Basic information on adrenal-gonad-pituitary interrelationships is discussed in this section.

The international units of gonadotrophins and sex hormones are given in the appendix. In addition, a list of the names, synonyms, and sources of various commercial hormone preparations, some of which are now obsolete, is given as an aid to understanding the past and present literature. There is a 35-page list of references arranged alphabetically under the names of the authors. Approximately 75 per cent of these references were published prior to 1940. If one should desire more detail than is given in the text, the bibliography will be found an adequate guide to additional information. An excellent 19-page index is given in the back of the book. There are 140 entries under estrogens; this gives some indication of the completeness of the index. The book is well printed on a good grade of paper.

This new edition of the *Biological Actions of the Sex Hormones* is a well-organized, clearly written, readable, and comprehensive review. It is chiefly concerned with the results of experimental work done on the vertebrates, particularly mammals. These results, which are enormous in quantity, are co-ordinated and clearly presented so as to give a concise but broad and rather detailed view of the basic information on sex hormones. Little consideration is given to unproved theories and postulations. The introductions at the beginning of several of the main sections give a historical perspective and serve to orient the reader. Another feature which will aid the reader is the 240 small tables summarizing many of the experimental results referred to in the text. There are certain omissions in the discussion of topics such as antigonadotrophins and the augmentation of gonadotrophins, but these are not of sufficient significance to detract materially from this monumental review.

It is the opinion of the reviewer that this book is indispensable as a reference for biology students who are interested in endrocrinology and also for professional endocrinologists. Although it is concerned chiefly with the results of experimental work, much of the information will be of value to the clinical endocrinologist. In addition, there is considerable material, such as that on the role of estrogens in mammary cancer, that will be of interest and value to those people who are working on growth and on cancer.

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Announcements

OAK RIDGE INSTITUTE OF NUCLEAR STUDIES

The Special Training Division of the Oak Ridge Institute of Nuclear Studies will conduct a 2-week course in instrumentation for radioisotope work from September 5 to 15, 1950. Emphasis will be on fundamental instrumentation for applied nuclear science problems.

The course will be open to chemical instrument specialists, electrical engineers, physicists, or others specializing in instrumentation. A knowledge of nuclear radiation properties and experience in electronic instrument design are prerequisites for attendance at the course, which will be limited to 40 participants.

One day each week will be spent on such subjects as proportional counters, construction practices, fast circuits, electrometers, scintillation counters, etc. Morning sessions will be devoted to a formal discussion of specific topics by a specialist in each field, with the afternoon sessions given over to informal discussions and group participation.

The courses will be conducted in the Institute's Training Building within the city of Oak Ridge. Security clearance is not required for access to this area. The building contains excellent laboratories for radioisotope training, a counting room, a well-stocked technical library and associated facilities for the conduction of courses of this nature. Specialists in various fields of instrumentation will supplement the Special Training Division staff in the conduction of the course.

A registration fee of $25, payable on arrival at Oak Ridge, is charged for the course. Participants will be expected to pay their own living and traveling expenses.

Additional information and application blanks may be obtained from Dr. Ralph T. Overman, Chairman, Special Training Division, Oak Ridge Institute of Nuclear Studies, P.O. Box 117, Oak Ridge, Tennessee.

NEW JOURNAL

The publication of a new German journal, *Archiv für Geschwulstforschung*, is announced by the publisher, Verlag Theodor Steinkopff, Loschwitzer Str. 32, Dresden-A53. The journal is edited by Dr. W. Friedrich (Berlin-Buch) and Dr. R. Schröder (Leipzig) and is devoted to papers on research, treatment, and statistical and organizational problems in oncology. Volume 1, 1949, comprises about 400 pages.